PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C07K 14/705, C12N 15/10, 15/12

A1

(11) International Publication Number: WO 98/38217

(43) Internati nal Publication Date: 3 September 1998 (03.09.98)

(21) International Application Number: PCT/US98/03991

(22) International Filing Date: 27 February 1998 (27.02.98)

(30) Priority Data:

60/039,465	27 February 1997 (27.02.97)	US
60/061,268	7 October 1997 (07.10.97)	US

(71)(72) Applicants and Inventors: TEITLER, Milt [US/US]; 24 Journey Lane, Glenmont, NY 12077 (US). HERRICK-DAVIS, Katharine [US/US]; 4020 Windsor Drive, Niskayuna, NY 12309 (US). EGAN, Christina, C. [US/US]; 91-H Kennsington Court, Guilderland, NY 12084 (US).

(74) Agent: WEINBERGER, Laurence; Suite 103, 882 Matlack Street, P.O. Box 1663, West Chester, PA 19380 (US).

(81) Designated States: AU, BR, CA, CN, CZ, IL, JP, KR, MX, NO, NZ, PL, RU, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: CONSTITUTIVELY ACTIVATED SEROTONIN RECEPTORS

(57) Abstract

Mutations have been discovered in mammalian G protein–coupled serotonin 5–HT_{2A} and 5–HT_{2C} receptors which render the mutated receptors constitutively active. An alignment methodology based on the highly conserved sixth transmembrane domain has been discovered for the monoamine receptors which accurately predicts the amino acid position in the third intracellular loop which, when mutated, produces constitutive activation of the receptor. Constitutive activation of the G protein–coupled serotonin receptors has been shown by the demonstration of an enhanced affinity and potency for serotonin, by increased basal activity of the second messenger system in the absence of agonist, and by reduction of the basal second messenger activity by inverse agonists.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	**ES**	Spain	**LS**	Lesotho	**SI**	Slovenia
AM	Armenia	**FI**	Finland	**LT**	Lithuania	**SK**	Slovakia
AT	Austria	**FR**	France	**LU**	Luxembourg	**SN**	Senegal
AU	Australia	**GA**	Gabon	**LV**	Latvia	**SZ**	Swaziland
AZ	Azerbaijan	**GB**	United Kingdom	**MC**	Monaco	**TD**	Chad
BA	Bosnia and Herzegovina	**GE**	Georgia	**MD**	Republic of Moldova	**TG**	Togo
BB	Barbados	**GH**	Ghana	**MG**	Madagascar	**TJ**	Tajikistan
BE	Belgium	**GN**	Guinea	**MK**	The former Yugoslav Republic of Macedonia	**TM**	Turkmenistan
BF	Burkina Faso	**GR**	Greece			**TR**	Turkey
BG	Bulgaria	**HU**	Hungary	**ML**	Mali	**TT**	Trinidad and Tobago
BJ	Benin	**IE**	Ireland	**MN**	Mongolia	**UA**	Ukraine
BR	Brazil	**IL**	Israel	**MR**	Mauritania	**UG**	Uganda
BY	Belarus	**IS**	Iceland	**MW**	Malawi	**US**	United States of America
CA	Canada	**IT**	Italy	**MX**	Mexico	**UZ**	Uzbekistan
CF	Central African Republic	**JP**	Japan	**NE**	Niger	**VN**	Viet Nam
CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

CONSTITUTIVELY ACTIVATED SEROTONIN RECEPTORS

The benefit of U.S. Provisional Application No. 60/039,465 filed February 27, 1997, and U.S. Provisional Application No. 60/061,268 filed October 7, 1997 is claimed for this application.

BACKGROUND OF THE INVENTION

Field Of The Invention

The present invention relates generally to the field of transmembrane receptors, more particularly to seven segment transmembrane G protein-coupled receptors, and most particularly to the serotonin (5-HT) receptors. Through genetic mutational techniques, the amino acid sequences of the native 5-HT_{2A} and 5-HT_{2C} receptors have been modified so that the receptors exist in a constitutively activated state exhibiting both a greater response to agonists and a coupling to the G Protein second messenger system even in the absence of agonist. A method for constitutively activating G protein-coupled 5-HT receptors in general is also disclosed.

Description Of Related Art

The research interest in G protein-coupled cell surface receptors has exploded in recent years as it has been apparent that variants of these receptors play a significant role in the etiology of many severe human diseases. These receptors serve a diverse array of signalling pathways in a wide variety of cells and tissue types. Indeed, over the past 20 years, G protein-coupled receptors have proven to be excellent therapeutic targets with the development of several hundred drugs directed towards activating or deactivating them.

G protein-coupled receptors form a superfamily of receptors which are related both in their structure and their function. Structurally the receptors are large macromolecular proteins embedded in and spanning the cell membrane of the receiving cell and are distinguished by a common structural motif. All the receptors have seven domains of between 22 to 24 hydrophobic amino acids forming seven α helixes arranged in a bundle which span the cell membrane substantially perpendicular to the cell membrane. The transmembrane helixes are joined by chains of hydrophilic amino acids. The amino terminal and three connecting chains extend into the extracellular environment while the carboxy

terminal and three connecting chains extend into the intracellular environment. Signalling molecules are believed to be recognized by the parts of the receptor which span the membrane or lie on or above the extracellular surface of the cell membrane. The third intracellular loop joining helixes five and six is thought to be the most crucial domain involved in receptor/G protein coupling and responsible for the receptor selectivity for specific types of G proteins.

Functionally, all the receptors transmit the signal of an externally bound signalling molecule across the cell membrane to activate a heterotrimeric transducing protein which binds GDP (guanosine diphosphate). Upon activation, the bound GDP is converted to GTP (guanosine triphosphate). The activated G protein complex then triggers further intracellular biochemical activity. Different G proteins mediate different intracellular activities through various second messenger systems including, for example, 3'5'-cyclic AMP (cAMP), 3'5'-cyclic GMP (cGMP), 1,2-diacylglycerol, inositol 1,4,5-triphosphate, and Ca^{2+}. Within the human genome, several hundred G protein-coupled receptors have been identified and endogenous ligands are known for approximately 100 of the group. While the seven transmembrane motif is common among the known receptors, the amino acid sequences vary considerably, with the most conserved regions consisting of the transmembrane helixes.

Binding of a signalling molecule to a G protein-coupled receptor is believed to alter the conformation of the receptor, and it is this conformational change which is thought responsible for the activation of the G protein. Accordingly, G protein-coupled receptors are thought to exist in the cell membrane in equilibrium between two states or conformations: an "inactive" state and an "active" state. In the "inactive" state (conformation) the receptor is unable to link to the intracellular transduction pathway and no biological response is produced. In the altered conformation, or "active" state, the receptor is able to link to the intracellular pathway to produce a biological response. Signalling molecules specific to the receptor are believed to produce a biological response by stabilizing the receptor in the active state.

Discoveries over the past several years have shown that G protein-coupled receptors can also be stabilized in the active conformation by means other than

binding with the appropriate signal molecule. Four principal methods have been identified: 1) molecular alterations in the amino acid sequence at specific sites; 2) stimulation with anti-peptide antibodies; 3) over-expression in in vitro systems; and 4) over-expression of the coupling G proteins. These other means simulate the stabilizing effect of the signalling molecule to keep the receptor in the active, coupled, state. Such stabilization in the active state is termed "constitutive receptor activation".

Several features distinguish the constitutively activated receptors. First, they have an affinity for the native signalling molecule and related agonists which is typically greater than that of the native receptors. Second, where several known agonists of varying activity (to the native receptor) were known, it was found that the greater the initial activity of the agonist, the greater was the increase in its affinity for the constitutively activated receptor. Third, the affinity of the constitutively activated receptor for antagonists is not increased over the affinity for the antagonist of the native receptor. Fourth, the constitutively activated receptors remain coupled to the second messenger pathway and produce a biological response even in the absence of the signalling molecule or other agonist.

The importance of constitutively activated receptors to biological research and drug discovery cannot be overstated. First, these receptors provide an opportunity to study the structure of the active state and provide insights into how the receptor is controlled and the steps in receptor activation. Second, the constitutively active receptors allow study of the mechanisms by which coupling to G proteins is achieved as well as how G protein specificity is determined. Third, mutated constitutively active receptors are now recognized in disease states. Study of constitutively activated receptors has demonstrated that many mutations may lead to constitutive activation and that a whole range of activation is possible.

Fourth, the existence of constitutively active receptors provides a novel screening mechanism with which compounds which act to increase or decrease receptor activity can be identified and evaluated. Such compounds may become lead compounds for drug research. Finally, studying the affect of classical antagonists

(compounds previously identified as, in the absence of agonist, binding to the receptor but causing no change in receptor activitiy, and, in the presence of agonist, competitively decreasing the activity of a receptor) and other drugs used as treatments on the constitutively active receptors has led to the discovery that there are compounds, inverse agonists, which decrease the constitutive activity of the active state of the receptors but which have no or little affect on the inactive state. The difference between antagonists, which act on the inactive state, and inverse agonists, which act on the active state, is only discernable when the receptor exhibits constitutive activity. These inverse agonists, identifiable with constitutively active receptors, present an entirely new class of potential compounds for drug discovery.

About 10 years ago, it was recognized that neurotransmitter receptors can be divided into two general classes depending on the rapidity of their response. Fast receptors were identified with ion channels and mediate millisecond responses while slower receptors were identified with G protein-coupled receptors. These G protein-coupled receptors include certain subtypes of the adrenergic as well as the muscarinic cholinergic (M1 - M5), dopaminergic (D1 - D5), serotonergic (5-HT1, 5-HT2, 5-HT4 - 5-HT7) and opiate (δ, κ, and μ) receptors. Each of these G protein-coupled neurotransmitter receptors has been associated with profound changes in mental activity and functioning, and it is believed that abnormal activity of these receptors may contribute to certain psychiatric disorders. Consequently, the elucidation of the mechanism of action of these receptors has been the focus of vigorous research efforts.

Serotonin receptors are of particular importance. Serotonin-containing cell bodies are found at highest density in the raphe regions of the pons and upper brain stem. However, these cells project into almost all brain regions and the spinal column. Serotonin does not cross the blood-brain barrier and is synthesized directly in neurons from L-tryptophan. In the CNS serotonin is thought to be involved in learning and memory, sleep, thermoregulation, motor activity, pain, sexual and aggressive behaviors, appetite, neuroendocrine regulation, and biological rhythms. Serotonin has also been linked to pathophysiological conditions such as anxiety, depression, obsessive-compulsive disorders,

schizophrenia, suicide, autism, migraine, emesis, alcoholism and neurodegenerative disorders. Presently several drugs are used to modify serotonin receptors: 1) 5-HT1: sumatriptan for treatment of migraine, ipsapirone and buspirone for treatment of anxiety; 2) 5-HT2: clozapine and risperidone for treatment of schizophrenia; and 3) 5-HT3: odanestron for the prevention of emesis in chemotherapy.

To date, fourteen serotonin receptors have been identified in 7 subfamilies based on structural homology, second messenger system activation, and drug affinity for certain ligands. The 5-HT_2 subfamily is divided into 3 classes: 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C}. 5-HT_{2A} and 5-HT_{2C} receptor antagonists are thought to be useful in treating depression, anxiety, psychosis, and eating disorders. 5-HT_{2A} and 5-HT_{2C} receptors exhibit 51% amino acid homology overall and approximately 80% homology in the transmembrane domains. The 5-HT_{2C} receptor was cloned in 1987 and led to the cloning of the 5-HT_{2A} receptor in 1990. Studies of the 5-HT_{2A} receptor in recombinant mammalian cell lines revealed that the receptor possessed two affinity states, high and low. Both the 5-HT_{2A} and 5-HT_{2C} receptors are coupled to phospholipase C and mediate responses through the phosphatidylinositol pathway. Studies with agonists and antagonists display a wide range of receptor responses suggesting that there is a wide diversity of regulatory mechanisms governing receptor activity. The 5-HT_{2A} and 5-HT_{2C} receptors have also been implicated as the site of action of hallucinogenic drugs.

Much of the knowledge about the structure of G protein-coupled receptors has come from the study of the β_2-adrenergic receptor. Over the last several years, site-directed mutagenesis has been used to try to determine the amino acid residues important for ligand binding in both the β_2-adrenergic and 5-HT_{2A} receptors. In addition, studies have suggested that in a native (inactive) state of G protein-coupled receptors, the third intracellular loop is tucked into the receptor and is not available for interaction with the G protein. A change of receptor conformation (active) results in the availability or exposure of the C-terminal region of the third intracellular loop.

In 1990 Cotecchia et al.[1] were studying the G protein specificity determining characteristics of the third intracellular loop by creating chimeric

receptors in which the third intracellular loops had been exchanged between the α_1-adrenergic receptor and the β_2-adrenergic receptor. The specific G protein coupled activation was essentially switched between the two receptors. While attempting to determine which portions of the loop were responsible for the specificity, Cotecchia et al. discovered an unexpected phenomena; namely that the modification in the third intracellular loop of the α_1-adrenergic receptor of three residues, Arg288, Lys290, and Ala293, created a mutant receptor with two orders of magnitude greater affinity for agonist and which coupled to the second messenger system even in the absence of agonist. These modifications were made in the carboxy end of the third cytoplasmic loop adjacent to the sixth transmembrane helix. The changes responsible for this increase were isolated to either a Ala293 → Leu or a Lys290 → His mutation. Thus, a constitutively active state of a G protein-coupled neuroreceptor had been created. Subsequently, Kjelsberg et al.[2] demonstrated that mutation of the amino acid at position 293 in the α_{1B}-adrenergic receptor to any other of the 19 amino acids also produced a constitutively active state. Subsequently, mutations in the β_2-adrenergic receptor near the carboxy end of the third cytoplasmic loop have also been shown by Samama et al.[3] to constitutively activate this receptor.

When foci resulting from constitutively active α_{1B}-adrenergic receptors were injected into nude mice, tumor formation occurred. Over the past 5 years, since the discovery that several thyroid adenomas contained mutations of the thyroid stimulating hormone (TSH) receptor, constitutively activated receptors have been found associated with several human disease states. The mutations responsible for these disease states have been found in the transmembrane domains and intracellular loops. For the TSH receptor, mutations at 13 different amino acid positions have been found in the transmembrane domain, the third intracellular loop, and the second and third extracellular loops. Clearly, constitutively activating mutations are not limited to the third intracellular loop and the critical site for constitutive activation varies with each G protein-coupled receptor. The importance of the initial observations was well stated in Cotecchia et al.[1]: "Such mutations might not only help to illuminate the biochemical mechanisms involved in receptor-G protein coupling but also provide models for how point mutations

might activate potentially oncogenic receptors."

In light of the above referenced discoveries, the importance and utility of discovering other constitutively activated neuronal receptors cannot be understated. However, the hope that other neuronal receptors could be easily and readily mutated to a constitutively active form by mutations in the third cytoplasmic loop was destroyed by the report of Burstein et al.[4] in 1995 of a comprehensive mutational approach to the G protein coupled M5 muscarinic acetylcholine receptor. In that approach, Burstein et al. had randomly and comprehensively mutated the C-terminal region of the third intracellular loop of the M5 muscarinic acetylcholine receptor, but no constitutive activating mutations were found.

Definition: CONSTITUTIVELY ACTIVATED RECEPTOR shall mean a G protein-coupled receptor which: 1) exhibits an increase in basal activity of the second messenger pathway in the absence of agonist above the level of activity observed in the wild type receptor in the absence of agonist; 2) may exhibit an increased affinity and potency for agonists; 3) exhibits an unmodified or decreased affinity for antagonists; and 4) exhibits a decrease in basal activity by inverse agonists.

SUMMARY OF THE INVENTION

Constitutively active forms of the rat 5-HT_{2A} and 5-HT_{2C} serotonin receptors have been obtained by a site-directed mutational method that will permit the constitutive activation of all mammalian G protein-coupled serotonin receptors. An amino acid position that will lead to a successful mutation in the serotonin receptor may be identified by alignment of the serotonin receptor against the amino acid sequence of the α_{1B}-adrenergic receptor. Mutating the amino acid in the serotonin receptor which corresponds to the most sensitive position in the α_{1B}-adrenergic receptor, alanine 293, yields a constitutively active serotonin receptor. A strongly constitutively active serotonin receptor is achieved when the mutation in the serotonin receptor is to one of the amino acids which produces the highest level of basal activation in constitutively activated α_{1B}-adrenergic receptors. Successful constitutive activation of the serotonin receptor can be shown by increased high basal levels of second messenger activity in the absence of agonist, increased affinity and potency for agonists, and an unmodified or

decreased affinity for antagonists. While standard methods of site-directed mutagenesis may be employed, the careful placement of restriction sites in the primer permits the more rapid and direct determination of the clone containing the desired mutated receptor.

It is the object of this invention to provide a general methodology for obtaining constitutively active forms of the G protein-coupled mammalian monoamine receptors.

It is a further object of this invention to provide a general methodology for obtaining constitutively active forms of the G protein-coupled mammalian serotonin receptors.

It is another object of this invention to provide a constitutively active 5-HT_{2A} serotonin receptor.

It is a further object of this invention to provide a constitutively active 5-HT_{2C} serotonin receptor.

Yet another object of this invention is to provide a method for rapidly identifying the clone containing the desired mutated receptor.

These and other achievements of the present invention will become apparent from the detailed description which follows.

DESCRIPTION OF THE FIGURES

Figure 1A shows the full DNA sequence for the rat 5-HT_{2A} serotonin receptor including the 5′ and 3′ untranslated regions with the translated codons underlined. Figure 1B shows the translated amino acid sequence for the rat 5-HT_{2A} receptor.

Figure 2A shows the full DNA sequence for the rat 5-HT_{2C} serotonin receptor including the 5′ and 3′ untranslated regions with the translated codons underlined. Figure 2B shows the translated amino acid sequence for the rat 5-HT_{2C} receptor.

Figure 3A shows the full DNA sequence for the rat α_{1B}-adrenergic receptor including the 5′ and 3′ untranslated regions with the translated codons underlined. Figure 3B shows the translated amino acid sequence for the rat α_{1B}-adrenergic receptor.

Figure 4 shows the amino acid sequences for part of the C-terminal third

intracellular loop and transmembrane domain VI for the 5-HT_{2A} and 5-HT_{2C} receptors aligned opposite the corresponding part of the α_{1b}-adrenergic receptor with numerals representing the amino acid positions in each receptor.

Figure 5 shows a schematic outline of the 5-HT_{2A} site-directed mutagenesis procedure.

Figure 6 shows a schematic outline of the 5-HT_{2C} site-directed mutagenesis procedure.

Figure 7 shows the competition curves of 5-HT for ^{3}H-ketanserin labeled native and mutant 5-HT_{2A} receptors. 0.5nM ^{3}H-ketanserin was used to label the native and mutant receptors transiently transfected in COS-7 cells.

Figure 8 shows the radioligand binding data of ^{3}H-ketanserin labeled native and mutant 5-HT_{2A} receptors in the presence of agonists and antagonists. 0.5 nM ^{3}H-ketanserin was used to label the native and mutant 5-HT_{2A} receptors expressed in COS-7 cells.

Figure 9 shows the stimulation of IP production in COS-7 cells expressing native or mutant 5-HT_{2A} receptors. IP production assays were performed using anion-exchange chromatography. The data are expressed as percent of maximal IP stimulation produced by 10 μM 5-HT.

Figure 10 shows the basal activity and 5-HT stimulation of the native and mutant 5-HT_{2A} receptors. IP levels were measured in COS-7 cells with vector alone, native 5-HT_{2A} receptors, or mutant 5-HT_{2A} receptors. The data are expressed as dpms of IP stimulation minus basal levels of IP produced by vector. Basal activity of vector alone was typically 400 dpms.

Figure 11 shows a saturation analysis of ^{3}H-ketanserin labeled native and cys → lys mutant receptors. Bmax values were determined by a BCA assay.

Figure 12 shows the competition curves of 5-HT for ^{3}H-mesulergine labeled native and mutant 5-HT_{2C} receptors. 1 nM ^{3}H-mesulergine was used to label the native and mutant receptors transiently transfected in COS-7 cells.

Figure 13 shows the radioligand binding analysis of native and mutant 5-HT_{2C} receptors. Native and mutant 5-HT_{2C} receptors expressed in COS-7 cells were labeled with 1 nM ^{3}H-mesulergine. 5-MT = 5-methoxytryptamine.

Figure 14 shows the 5-HT stimulation of IP production in COS-7 cells

transfected with the ser → lys or ser → phe mutated receptors. Cells were labeled with ^{3}H-myoinositol and challenged with 5-HT (0.1 nM - 10 nM). Total IP production was measured by anion exchange chromatography.

Figure 15 shows the EC_{50} values for the 5-HT stimulation of IP production in COS-7 cells transfected with native, mutant ser → lys receptor, and mutant ser → phe receptor. Figure 15 also shows the results of ^{3}H-mesulergine saturation analyses. Saturation experiments were performed using ^{3}H-mesulergine (0.1 nM - 5.0 nM).

Figure 16 shows the effect of the ser → lys and ser → phe mutations on basal levels of IP production by the mutated 5-HT_{2C} receptors. IP levels were measured in COS-7 cells with vector alone, native 5-HT_{2C} receptors, or mutant 5-HT_{2C} receptors. The data are expressed as dpms of IP stimulation minus basal levels of IP produced by vector.

Figure 17 shows the inverse agonist activity of spiperone and ketanserin on the mutated constitutively active 5-HT_{2A} cys → lys receptor. Parallel transfections with the native 5-HT_{2A} receptor were performed to determine native basal activity which was then subtracted from the mutant receptor basal activity to determine constitutive stimulation.

Figure 18 shows the inverse agonist activity of chlorpromazine, haloperidol, loxapine, spiperone, clozapine and risperidone on the mutated constitutively active 5-HT_{2A} cys → lys receptor.

Figure 19 shows the inverse agonist activity of mianserin and mesulergine on the mutated constitutively active 5-HT_{2C} ser → lys receptor both in the presence and absence of 5-HT.

Figure 20A sets forth the full DNA sequence for the human 5-HT_{2A} serotonin receptor with the translated codons underlined. The sixth transmembrane domain conserved sequence of WxPFFI is indicated with block letters. Figure 20B shows the translated amino acid sequence for the human 5-HT_{2A} receptor.

Figure 21A sets forth the full DNA sequence for the human 5-HT_{2C} serotonin receptor with the translated codons underlined. The sixth transmembrane domain conserved sequence of WxPFFI is indicated with block

letters. Figure 21B shows the translated amino acid sequence for the human 5-HT_{2C} receptor.

Figure 22 is the amino acid sequence of the 5-HT_{2A} cys → lys mutant receptor with the mutated amino acid shown as a larger outlined letter.

Figure 23 is the DNA sequence of the 5-HT_{2A} cys → lys mutant receptor including the 5′ and 3′ untranslated regions with the translated codons underlined. The bases specifying the #322 lysine mutant are shown as larger outlined letters, and the starting and ending locations of the primer are also indicated.

Figure 24 is the DNA sequence of the 5-HT_{2A} cys → lys mutant receptor including the 5′ and 3′ untranslated regions with the translated codons underlined. The bases specifying the #322 lysine mutant are shown as larger outlined letters, and the starting and ending locations of the primer are also indicated. In addition the two bases which were mutated to create the Sca1 site are shown as larger outlined letters and are indicated with arrows.

Figure 25 is the amino acid sequence of the 5-HT_{2A} cys → arg mutant receptor with the mutated amino acid shown as a larger outlined letter.

Figure 26 is the DNA sequence of the 5-HT_{2A} cys → arg mutant receptor including the 5′ and 3′ untranslated regions with the translated codons underlined. The bases specifying the #322 arginine mutant are shown as larger outlined letters, and the starting and ending locations of the primer are also indicated.

Figure 27 is identical to Figure 26 since the AGG mutation introduced for arginine creates an Mnl1 restriction site by itself at #319.

Figure 28 is the amino acid sequence of the 5-HT_{2A} cys → glu mutant receptor with the mutated amino acid shown as a larger outlined letter.

Figure 29 is the DNA sequence of the 5-HT_{2A} cys → glu mutant receptor including the 5′ and 3′ untranslated regions with the translated codons underlined. The bases specifying the #322 glutamic acid mutant are shown as larger outlined letters, and the starting and ending locations of the primer are also indicated.

Figure 30 is the DNA sequence of the 5-HT_{2A} cys → glu mutant receptor

including the 5′ and 3′ untranslated regions with the translated codons underlined. The bases specifying the #322 glutamic acid mutant are shown as larger outlined letters, and the starting and ending locations of the primer are also indicated. In addition the additional base which was mutated to create the Rsa1 site is shown as a larger outlined letter and is indicated with an arrow.

Figure 31 is the amino acid sequence of the 5-HT_{2C} ser → lys mutant receptor with the mutated amino acid shown as a larger outlined letter.

Figure 32 is the DNA sequence of the 5-HT_{2C} ser → lys mutant receptor including the 5′ and 3′ untranslated regions with the translated codons underlined. The bases specifying the #312 lysine mutant are shown as larger outlined letters, and the starting and ending locations of the primer are also indicated.

Figure 33 is the DNA sequence of the 5-HT_{2C} ser → lys mutant receptor including the 5′ and 3′ untranslated regions with the translated codons underlined. The bases specifying the #312 lysine mutant are shown as larger outlined letters, and the starting and ending locations of the primer are also indicated. In addition the base which was mutated to create the Sca1 site is shown as a larger outlined letter and is indicated with an arrow.

Figure 34 is the amino acid sequence of the 5-HT_{2C} ser → phe mutant receptor with the mutated amino acid shown as a larger outlined letter.

Figure 35 is the DNA sequence of the 5-HT_{2C} ser → phe mutant receptor including the 5′ and 3′ untranslated regions with the translated codons underlined. The bases specifying the #312 phenylalanine mutant are shown as larger outlined letters, and the starting and ending locations of the primer are also indicated.

Figure 36 is the DNA sequence of the 5-HT_{2C} ser → phe mutant receptor including the 5′ and 3′ untranslated regions with the translated codons underlined. The bases specifying the #312 phenylalanine mutant are shown as larger outlined letters, and the starting and ending locations of the primer are also indicated. In addition the base which was mutated to create the Sca1 site is shown as a larger outlined letter and is indicated with an arrow.

DETAILED DESCRIPTION OF THE INVENTION

Despite the disappointing results obtained by Burstein in mutating positions in the third intracellular loop of the M5 muscarinic acetylcholine receptor, the present inventive efforts focused on finding mutations at the carboxy end of the third intracellular loop near the sixth transmembrane helix in the serotonin receptors. DNA and amino acid sequences for rat 5-HT_{2A} and 5-HT_{2C} serotonin receptors were obtained from GeneBank as was the DNA and amino acid sequence for the α_{1B}-adrenergic receptor. Figures 1, 2, and 3 list the full DNA and translated amino acid sequences for these receptors.

Receptor Alignment:

As noted above, Cotecchia et al. had identified amino acid position number 293 in the third intracellular loop adjoining the sixth transmembrane domain in the α_{1B}-adrenergic receptor as a critical position, mutation of which lead to constitutive activity. However, the length of the serotonin receptors is different than the α_{1B}-adrenergic receptor, and even had they been the same, matching the ends would not necessarily provide a structural or functional match. What was important was to find an alignment method which made sense in terms of locating the equivalent functional site to position 293 of the α_{1B}-adrenergic receptor in the serotonin receptors.

A meaningful alignment method has been discovered based upon the fact that the transmembrane domains are highly conserved in G protein-coupled receptors. A series of conserved amino acid positions were identified in the sixth transmembrane domain which permit alignment of the transmembrane domain and the adjacent third intracellular loop between receptors. In Figure 5 the conserved sixth transmembrane domain amino acid sequence WxPFFI (x may be variable) has been used to align the three receptors. Alignment using this sequence also aligns the LGIV sequence found at the intracellular beginning of the sixth transmembrane domain which is connected to the third intracellular loop. This alignment indicates that in the 5-HT_{2A} receptor the cysteine at position #322 corresponds to the alanine at position #293 in the α_{1B}-adrenergic receptor. In the 5-HT_{2C} receptor, the corresponding amino acid is a serine at position #312.

It should be noted that position 293 is not the only position in the α_{1B}-

adrenergic receptor which, when mutated, produced a constitutively active receptor. While Cotecchia et al.[1] reported that the A293L mutation produced the greatest constitutive activation, they also noted that the K290H mutation also induced dramatic constitutive activity. There are clearly other sites in the third intracellular loop of each of these receptors that can be mutated. In the future, other sites on other receptors may be reported. However, the alignment methodology presented above should serve to permit the structural correlation between different receptors so that information gleaned from one receptor may be utilized to mutate another receptor. However, the evidence presently available suggests that the third position removed from the beginning of the transmembrane domain represented by position 293 in the α_{1B}-adrenergic receptor seems to play a crucial role in the binding and activation of the coupled G protein, and that mutations introduced at that position alter the tertiary structure of the region.

As noted earlier, Kjelsberg et al.[2] further demonstrated that substitution of any of the 19 amino acids at position 293 of the α_{1B}-adrenergic receptor produced constitutive activity. However, the relative activity increased in the following order of amino acids: S, N, D, G, T, H, W, Y, P, V, L, M, Q, I, F, C, R, K, and E. In that study, replacing the native amino acid with amino acids having long basic or acidic side chains produced the greatest degree of constitutive activity, while amino acids with aromatic substituents produced an intermediate degree of constitutive activity. It is proposed that this order, with minor variations, exists for most G protein-coupled receptors due to the importance of the third position removed from the beginning of the transmembrane domain. A reasonable starting place for mutating receptors should therefore involve mutation to one of the amino acids at the most active end of the above list. Further, the tertiary structure of the region may be significantly altered by substituting an amino acid with longer side chains or of different polarity from the native amino acid.

Efficient Screening of Mutant Receptors:

When performing site-directed mutagenesis, it is common (and necessary) laboratory practice to fully sequence the cloned receptor to confirm that the mutation has been incorporated. However, because colonies containing the

mutant receptor cannot be distinguished from those that do not, it is necessary to sequence each colony. A method, outlined schematically by way of example in Figure 5 for the 5-HT_{2A} cys → lys receptor mutation and in Figure 6 for the 5-HT_{2C} ser → lys and ser →phe receptor mutations, has been devised that rapidly and easily eliminates most non-mutated colonies, and from those remaining, identifies the mutant colony so that unnecessary sequencing is avoided. A two-pronged approach is used. The first prong is designed to prevent non-mutated vector from being incorporated during the first transformation by digesting the vector. E coli will only incorporate uncut (circular) plasmid DNA. Recognizing the limitations of the first prong, namely, that all restriction digests are not 100% complete so that some of the colonies at the end of the procedure will contain native DNA instead of mutant DNA, the second prong is designed to easily identify among the remaining colonies, those colonies containing the desired mutation after a second transformation.

To begin, a unique restriction site, not occurring in the native amino acid sequence, is incorporated into the mutant. It is possible to introduce the unique restriction site because of the degeneracy of the genetic code. The unique restriction site is ideally located within or near the amino acid(s) which specify the structural mutation which is being introduced into the mutant. Thus, the restriction site can be located on the same mutagenic primer as the structural mutation.

In addition, during the initial annealing, a second primer is used to remove a restriction site specific to the vector being used. When the second strand is synthesized with polymerase and ligase, only the second strand of the vector (the one not containing the mutations) will contain the original vector restriction site. Subsequently, after transformation, the colonies can be treated with the restriction enzyme specific for the vector site and only those resulting from the wildtype vector will be digested. Digested (cut) DNA will not be taken up by E. coli during the second transformation step. The colonies containing the mutated vector will not be digested and will be taken up by E. coli during the final transformation step.

Each resulting colony can be tested to see whether the restriction enzyme,

which recognizes the unique site introduced by the mutated primer, digests the DNA. Only samples from colonies containing the desired mutation will be digested. These colonies can then be sequenced to confirm the insertion of the mutated amino-acid. It is unnecessary to sequence colonies whose DNA is not digested by the restriction enzyme. This procedure yields a much more highly efficient method by saving both time and expense of sequencing every colony which results from the transformation experiment.

Measurement of Receptor-Coupled Second Messenger Activation:

In order to measure the stimulation produced through the 5-HT_{2A} and the 5-HT_{2C} receptors, an assay was utilized which measures the accumulation of inositol phosphates, the product that is formed when phosphatidylinositol 4,5-bisphosphate is hydrolyzed to DAG and IP. This assay was established by Berridge and coworkers (1983) in studies of the blowfly salivary glands, and found to be an accurate measurement of the stimulation of phospholipase C through receptor activation. ^{3}H-myoinositol is incorporated into the cell membrane by conversion to phosphatidylinositol 4,5-bisphosphate and upon receptor activation, is cleaved by phospholipase C to yield two products: diacylglycerol and ^{3}H-inositol 1,4,5 triphosphate (IP_3).

Inositol-free media must be used for this assay because unlabeled inositol, which is normally found in many commercially available media, can result in less than maximal incorporation of radiolabeled inositol into the cell membrane, resulting in a reduction in the amount of ^{3}H-IP that would be detected. The ^{3}H-IP is recovered by anion-exchange chromatography in which IP is separated from anion-exchange resin using washes of increasing concentrations of formate.

IP_3 is rapidly hydrolyzed to IP_2 by an inositol triphosphatase which is then converted to IP by inositol bisphosphatase. Because IP_3 is hydrolyzed so quickly, accumulation of IP would be hard to measure unless the cycle of IP to inositol and phosphate is blocked. Lithium is used in this assay to block the enzyme which converts IP to inositol and phosphate (myo-inositol monophosphatase). This ensures that IP levels can accumulate and be experimentally measured and are not undergoing the normal rapid degradation pathway. These experiments are also performed in serum free media in order to remove serotonin that can be found in

serum which would complicate experimental results.

The total IP levels were measured in order to obtain an accurate measurement of the total amount of stimulation that occurred. The actual experimental conditions and concentrations of reagents used in this assay are set forth in the methods and materials sections under each example below.

Example 1: Constitutive Activation Of The 5-HT_{2A} Receptor:

Three separate mutations of the 5-HT_{2A} receptor were made. The cysteine at position 322 was mutated to lysine, glutamate, and arginine.

Materials and Methods For Site-directed Mutagenesis:

The rat 5-HT2A receptor cDNA was ligated into the mammalian expression vector pcDNA3 (Invitrogen) using EcoR1 (GIBCO). This construct served as the native template for site-directed mutagenesis performed using Clontech's transformer kit. Mutagenic primers (Midland Certified Reagent Company) were designed as follows: the C322K primer was complementary to amino acid nos. 318-329 of the native 5-HT2A cDNA, while changing amino acid no. 322 from cysteine (TGC) to lysine (AAG). The same primer was designed to incorporate a Sca1 restriction site using amino acid nos. 323 and 324 by changing the third base in amino acid no. 323, lysine, from AAG to AAA and the third base in amino acid no. 324, valine from GTG to GTA. The C322E and C322R were designed complementary to amino acid nos. 319-330 of the native 5-HT2A cDNA, while changing amino acid no. 322 from cysteine (TGC) to glutamate (GAG) and arginine (AGG). In the C322E primer, an Rsa1 site was introduced by changing the third base in amino acid no. 324, valine, from GTG to GTA. The C322R mutation in the primer created an Mnl1 site, by itself, at amino acid no. 319. The selection primer, complementary to bases 4,871-4,914 of the pcDNA3 vector, was designed to remove a unique PVUI site by changing base G to T at location 4891. Phosphorylated primers were annealed to 10 ng of alkaline-denatured plasmid template by heating to 65°C for 5 min and cooling slowly to 37°C. Mutant DNA was synthesized using T4 DNA polymerase and ligase (Clontech) by incubating for 1 hr at 37°C, followed by digestion with PVU1 (GIBCO) and transformation of BMH71-18mutS E. coli (Clontech). Plasmid was purified using the Wizard miniprep kit (Promega), digested with PVU1, and used to transform

DH5(E.Coli (GIBCO). Individual colonies were isolated and plasmid DNA was digested with SCA1, Mnl1 or Rsa1 to screen for C322K, C322E and C322R mutations, respectively (GIBCO). DNA sequencing (Sequenase version 2.1 kit,USB, ^{35}Sd-ATP, New England Nuclear) was performed to confirm the incorporation of lysine, glutamate, or arginine at amino acid no. 322. Sequencing reactions were run on a 5% acrylamide/bis (19:1) gel (Bio-Rad) for 2 hr at 50°C, dried for 2 hr at 80°C, and exposed on Kodak Biomax MR film for 24 hr at –80°C.

In Figure 22 is shown the amino acid sequence of the 5-HT_{2A} cys → lys mutant receptor with the mutated amino acid shown as a larger outlined letter. Figure 23 shows the resulting DNA sequence of the 5-HT_{2A} cys → lys mutant receptor including the 5′ and 3′ untranslated regions with the translated codons underlined. The bases specifying the #322 lysine mutant are shown as larger outlined letters, and the starting and ending locations of the primer are also indicated. In addition to showing the mutated DNA sequence of the 5-HT_{2A} cys → lys mutant receptor, Figure 24 shows the two bases, which were mutated to create the Sca1 site, as larger outlined letters and are indicated with arrows.

In Figure 25 is shown the amino acid sequence of the 5-HT_{2A} cys → arg mutant receptor with the mutated amino acid shown as a larger outlined letter. Figure 26 shows the resulting DNA sequence of the 5-HT_{2A} cys → arg mutant receptor including the 5′ and 3′ untranslated regions with the translated codons underlined. The bases specifying the #322 arginine mutant are shown as larger outlined letters, and the starting and ending locations of the primer are also indicated. Figure 27 showing the added restriction site is identical to Figure 26 since the arginine mutation to AGG creates, by itself, an Mnl1 restriction site at #319.

In Figure 28 is shown the amino acid sequence of the 5-HT_{2A} cys → glu mutant receptor with the mutated amino acid shown as a larger outlined letter. Figure 29 shows the resulitng DNA sequence of the 5-HT_{2A} cys → glu mutant receptor including the 5′ and 3′ untranslated regions with the translated codons underlined. The bases specifying the #322 glutamic acid mutant are shown as larger outlined letters, and the starting and ending locations of the primer are also indicated. Figure 30 shows the additional base mutation introduced in amino

acid 324 to create an Rsa1 site. The base mutation is indicted by a larger outlined letter and an arrow.

Cell culture and transfection:

COS-7 cells were grown in Dulbecco's modified Eagle's medium (DMEM, Sigma) with 10% fetal bovine serum (Sigma) in 5% CO_2 at 37°C and subcultured 1:8 twice a week. Twenty-four hours before transfection, cells were seeded at 30% confluence in 100-mm dishes for radioligand binding assays or at 10^5 cells per well in 24-well cluster plates for IP production assays. Cells were transfected with native or mutant 5-HT2A cDNA using Lipofectamine (GIBCO). This was accomplished by combining 20 μl of Lipofectamine with 2.5 μg of plasmid per 100-mm dish or 2 μl of Lipofectamine with 0.25 μg of plasmid per well. Transfections were performed in serum-free DMEM for 4 hr at 37°C.

Radioligand binding:

Thirty-six hours after transfection, membranes were prepared from COS-7 cells by scraping and homogenizing in 50mM Tris-HCl/5mM $MgCl_2$/0.5mM EDTA, pH 7.4 (assay buffer), and centrifugation at 10,000xg for 30 min. Membranes were resuspended in assay buffer, homogenized, and centrifuged again. After resuspension in assay buffer, 1-ml membrane aliquots (approximately 10 μg of protein measured by bicinchoninic acid assay) were added to each tube containing 1ml of assay buffer with 0.5nM [^{3}H] ketanserin and competing drugs. 10μM spiperone was used to define non-specific binding. Saturation experiments were performed by using [^{3}H]ketanserin (0.1-5.0nM). Samples were incubated at 23°C for 30 minutes, filtered on a Brandel cell harvester, and counted in Ecoscint cocktail (National Diagnostics) in a Beckman liquid scintillation counter at 40% efficiency.

Phosphatidylinositol hydrolysis:

Inositol phosphate (IP) production was measured using a modified combination of the methods of Berridge et al. (1982) and Conn and Sanders-Bush (1985). In brief, 24 h after transfection, cells were washed with phosphate-buffered saline (PBS) and labeled with 0.25 μCi/well of myo-[^{3}H]inositol (New England Nuclear) in inositol free/serum-free DMEM (GIBCO) for 12 h at 37°C. HPLC analysis of this culture medium, after incubation, has been reported to

contain $<10^{-10}$M 5-HT (Barker et al. 1994). After labeling, cells were washed with PBS and preincubated in inositol-free/serum-free DMEM with 10mM LiCl and 10μM pargyline (assay medium) for 10 min at 37°C. When antagonists were used, they were added during the 10-min preincubation period. 5-HT (Sigma), or assay medium alone, was added to each well and incubation continued for an additional 35 min (Westphal et al., 1995). Assay medium was removed and cells were lysed in 250 μl of stop solution (1 M KOH/18mM sodium borate/3.8mM EDTA) and neutralized by adding 250 μl of 7.5 % HCl. The contents of each well were extracted with 3 volumes of chloroform/methanol (1:2), centrifuged 5 min at 10,000xg, and the upper layer loaded onto a 1-ml AG1-X8 resin (100-200 mesh, Bio-Rad) column. Columns were washed with 10ml of 5 mM myo-inositol and 10ml of 5 mM sodium borate/60mM sodium formate. Total IPs were eluted with 3ml of 0.1 M formic acid/1 M ammonium formate. Radioactivity was measured by liquid scintillation counting in Ecoscint cocktail.

Demonstration of Constitutive Activation:

Constitutive activity of the mutated 5-HT_{2A} receptors is demonstrated by the fact that the mutated receptors exhibit all the hallmark characteristics established for constitutive activation: a showing of increased agonist affinity, increased agonist potency, and coupling to the G protein second messenger system in the absence of agonist.

Figure 7 shows the competition curves of 5-HT for ^{3}H-ketanserin labeled native and mutant 5-HT_{2A} receptors. 0.5nM ^{3}H-ketanserin was used to label the native and mutant receptors transiently transfected in COS-7 cells. While the native receptor demonstrated a relatively low affinity for 5-HT (K_i = 293 nM), the three mutant receptors displayed a high affinity for 5-HT with the cys → lys mutant exhibiting a 12-fold increase in affinity for 5-HT (K_i = 25 nM), the cys → arg mutant exhibiting a 27-fold increase in affinity for 5-HT (K_i = 11 nM). and the cys → glu mutant exhibiting a 3.4-fold increase in affinity for 5-HT (K_i = 86 nM).

To determine whether other agonists would display a similar increase in affinity for the mutant receptors, two known agonists (DOM and DOB) were tested with both the native and cys → lys mutant. Figure 8 shows the radioligand binding data of ^{3}H-ketanserin labeled native and mutant 5-HT_{2A} receptors in the

presence of agonists and antagonists. 0.5 nM ^{3}H-ketanserin was used to label the native and mutant 5-HT_{2A} receptors expressed in COS-7 cells. The DOM and DOB agonists show increased affinity for the mutant receptor, as is seen for 5-HT. The K_i for DOM shows a 5-fold increase, while the K_i for DOB shows a 7.4-fold increase.

To determine if the mutant 5-HT_{2A} receptors would exhibit an increase in agonist potency relative to the native 5-HT_{2A} receptor, 5-HT stimulation of the native and mutant 5-HT_{2A} receptors was measured using an IP production assay. Figure 9 shows the stimulation of IP production in COS-7 cells expressing native or mutant 5-HT_{2A} receptors. Both the cys → lys and cys → glu mutant receptor curves exhibit a leftward shift away from the native curve in the 5-HT dose-response indicating that there was an increase in 5-HT potency at the mutant receptors. The cys → lys and cys → glu mutant receptors displayed EC_{50} values of 25 nM and 61 nM, respectively, as compared to the native 5-HT_{2A} receptor which had an EC_{50} value of 152 nM.

Figure 10 shows the basal activity and 5-HT stimulation of the native and mutant 5-HT_{2A} receptors. As can be seen, both the cys → lys and the cys → glu mutant 5-HT_{2A} receptors show dramatic increases in basal intracellular inositol phosphate (IP) accumulation compared to the native receptor. The cys → lys mutant receptor produced a 345% (8-fold) increase in IP levels over the vector control. The cys → glu mutant receptor produced a 158% (3.7-fold) increase in IP levels over the vector control. Upon the addition of 10 μM 5-HT, both the native and mutant receptors produced an additional increase in IP production. The basal activity of the cys → lys mutant was 48% of that of the maximally stimulated native 5-HT_{2A} receptor. The basal activity of the cys → glu mutant was 31% of that of the maximally stimulated native 5-HT_{2A} receptor.

In order to determine whether the above results were due to an increase in the number of expressed mutant receptors rather than to a change in the properties of the mutated receptors, saturation curves were generated. Figure 11 shows a saturation analysis of ^{3}H-ketanserin labeled native and cys → lys mutant receptors. B_{MAX} values were determined by a BCA assay. For the native receptor the B_{MAX} = 193 +/- 37 fmol/mg, while for the cys → lys mutant receptor, the

B_{MAX} = 218 +/- 31 fmol/mg. There is no significant difference in the B_{MAX} values for the native and mutant receptors. The K_D of ^{3}H-ketanserin also did not differ between the native and mutant receptors. These data demonstrate that the results were not due to an increase in number of expressed mutant receptors compared to expressed native receptors.

Thus, the mutated 5-HT_{2A} receptors meet all the criteria for constitutively activated receptors; they show a higher affinity for agonists; they show a higher potency for 5-HT; and they show activation (coupling) of the G protein second messenger pathway (IP production) even in the absence of agonist.

Example 2: Constitutive Activation of 5-HT_{2C} Receptor

Materials and Methods For Site-directed Mutagenesis:

The rat 5-HT_{2C} receptor cDNA was ligated into the mammalian expression vector pcDNA3 (Invitrogen) using BamHI (Gibco). This construct served as the native template for site-directed mutagenesis performed using Clonetech's Transformer kit. Mutagenic primers (Midland Certified Reagent Company) were designed complementary to amino acids #308-317 of the native 5-HT_{2C} cDNA, while changing amino acid #312 from serine (TCC) to lysine (AAG) or phenylalanine (TTC). The same primers were designed to incorporate an Sca1 restriction site at amino acid #314 by changing the third codon in valine from GTC to GTA. The selection primer, complementary to bases 2081-3017 of the pcDNA3 vector, was designed to remove a unique Sma1 site by changing glycine at base 2093 from GGG to GGA. Phosphorylated primers were annealed to 10ng of alkaline denatured plasmid template by heating to 65°C for 5 minutes and cooling slowly to 37°C. Mutant DNA was synthesized using T4 DNA polymerase and ligase (Clonetech) by incubating for 1 hour at 37°C, followed by digestion with Sma1 (Gibco) and transformation of BMH71-18mutS E. coli (Clonetech). Plasmid was purified using the Wizard miniprep kit (Promega), digested with Sma1, and used to transform DH5α E. coli (Gibco). Individual colonies were isolated and plasmid DNA was digested with Sca1 to screen for S312K and S312F mutants (Gibco). S312K and S312F mutant plasmids contain an additional Sca1 site and appear as two bands (2.3Kb and 7.6Kb) when run on a 1% agarose gel. DNA sequencing (Sequenase version 2.1 kit USB, ^{35}Sd-ATP NEN)

was performed to confirm the incorporation of lysine or phenylalanine at amino acid #312. Sequencing reactions were run on a 5% acrylamide/bis (19:1) gel (BioRad) for 2 hours at 50°C, dried for 2 hours at 80°C, and exposed to Kodak Biomax MR film for 24 hours at -80°C.

In Figure 31 is shown the amino acid sequence of the 5-HT_{2C} ser → lys mutant receptor with the mutated amino acid shown as a larger outlined letter. Figure 32 shows the resulting DNA sequence of the 5-HT_{2C} ser → lys mutant receptor including the 5′ and 3′ untranslated regions with the translated codons underlined. The bases specifying the #312 lysine mutant are shown as larger outlined letters, and the starting and ending locations of the primer are also indicated. In addition to showing the mutated DNA sequence of the 5-HT_{2C} ser → lys mutant receptor, Figure 33 shows the base, which was mutated to create the Sca1 site, as a larger outlined letter indicated with an arrow.

In Figure 34 is shown the amino acid sequence of the 5-HT_{2C} ser → phe mutant receptor with the mutated amino acid shown as a larger outlined letter. Figure 35 shows the resulting DNA sequence of the 5-HT_{2C} ser → phe mutant receptor including the 5′ and 3′ untranslated regions with the translated codons underlined. The bases specifying the #312 phenylalanine mutant are shown as larger outlined letters, and the starting and ending locations of the primer are also indicated. In addition to showing the mutated DNA sequence of the 5-HT_{2C} ser → phe mutant receptor, Figure 36 shows the base, which was mutated to create the Sca1 site, as a larger outlined letter indicated with an arrow.

Cell culture and transfection:

COS-7 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM, Sigma) with 10% fetal bovine serum (Sigma) in 5% CO_2 at 37°C and subcultur d 1:8 twice a week. Twenty-four hours prior to transfection, cells were seeded at 30% confluence in 100mm dishes for radioligand binding assays or at 10^5 cells/well in 24 well cluster plates for PI assays. Cells were transfected with native or mutant 5-$HT2_c$ cDNA using Lipofectamine (Gibco). This was accomplished by combining 20 μl of lipofectamine with 2.5 μg plasmid per 100mm dish or 2 μl lipofectamine and 0.25 μg plasmid per well. Transfections were performed in serum-free DMEM for 4 hours at 37°C.

Radioligand binding:

Thirty-six hours after transfection, membranes were prepared from COS-7 cells by scraping and homogenizing in 50mM Tris-HCl / 5mM $MgCl_2$ / 0.5mM EDTA pH 7.4 (assay buffer) and centrifugation at 10,000xg for 30 minutes. Membranes were resuspended in assay buffer, homogenized and centrifuged again. Following resuspension in assay buffer, 1 ml membrane aliquots (approximately 10 μg protein measured by BCA assay) were added to each tube containing 1ml of assay buffer with 1nM ^{3}H-mesulergine and competing drugs. 10μM mianserin was used to define non-specific binding. Saturation experiments were performed using ^{3}H-mesulergine (0.1nM-5.0nM) or ^{3}H-5-HT (0.1nM-30nM) in the absence of presence of 10μM GppNHp (RBI). Samples were incubated at 37°C for 30 minutes, filtered on a Brandel cell harvester, and counted in Ecoscint cocktail (National Diagnostics) in a Beckman liquid scintillation counter at 40% efficiency.

Phosphatidylinositol hydrolysis:

Inositol phosphate (IP) production was measured using a modified combination of the methods of Berridge et al., 1982 and Conn and Sanders-Bush 1985. Briefly, 24 hours after transfection, cells were washed with PBS and labeled with 0.25μCi/well of ^{3}H-myoinositol (NEN) in inositol-free/serum-free DMEM (Gibco) for 12 hours at 37°C. Following labeling, cells were washed with PBS and preincubated in inositol-free/serum-free DMEM with 10mM LiCl and 10μM pargyline (assay medium) for 10 minutes at 37°C. When antagonists were used they were added during the 10 minute preincubation period. 5-HT (Sigma), or assay medium alone, was added to each well and incubation continued for an additional 35 minutes (Westphal et al., 1995). Assay medium was removed and cells were lysed in 250 μl of stop solution (1M KOH / 18mM NaBorate / 3.8mM EDTA) and neutralized by adding 250μl of 7.5% HCl. The contents of each well were extracted with 3 volumes of chloroform:methanol (1:2), centrifuged 5 minutes at 10,000xg, and the upper layer loaded onto a 1ml AG1-X8 resin (100-200 mesh, BioRad) column. Columns were washed with 10mls of 5mM myoinositol and 10mls f 5mM NaBorate / 60mM NaFormate. Total IPs were eluted with 3mls of 0.1M formic acid / 1M ammonium formate. Radioactivity was

measured by liquid scintillation counting in Ecoscint cocktail.

Stable Transfection:

Although not yet fully characterized, it has been found possible to create a stable cell line expressing mutant receptors by the following method. The rat 5-HT_{2C} cDNA (edited VSI isoform) was used as a template for site-directed mutagenesis to convert amino acid 312 from serine to lysine as previously described. Native and S312K 5-HT_{2C} cDNAs were ligated into the BamHI/EcoRI site of the pZeoSV2+ mammalian expression vector (Invitrogen) containing the zeocin resistance gene. NIH3T3 cells (ATCC) were stably transfected using the high efficiency BES method. Briefly, cells were seeded at $5x10^5$ cells/100mm culture dish in complete medium (DMEM/10%FBS) and grown in 5% CO_2 at 37° overnight. Twenty micrograms of pZeoSV2/5-HT_{2C} DNA (linearized with BglII) was mixed with 500μl of 0.25M $CaCl_2$ and 500μl of 2x BES solution (50mM N,N-bis-2-hydroxyethyl-2-aminoethanesulfonic acid; 280mM NaCl; 1.5mM Na_2HPO_4; pH to *6.95)* and incubated at 25°C for 20 minutes. The solution was added dropwise on top of the cells. The cells were incubated for 20 hours at 35°C in 3% CO_2, washed twice with PBS, complete medium replenished, and incubated for 48 hours at 37°C in 5% CO_2. Cells were split 1:4 into complete medium containing 500μg/ml zeocin. Individual colonies were isolated and tested for 5-HT_{2C} receptor expression by ^{3}H-mesulergine binding.

Demonstration of Constitutive Activation:

Constitutive activity of the mutated 5-HT_{2C} receptors is demonstrated by the fact that the mutated receptors also exhibit all the hallmark characteristics established for constitutive activation: a showing of increased agonist affinity, increased agonist potency, and coupling to the G protein second messenger system in the absence of agonist.

Figure 12 shows the competition curves of 5-HT for ^{3}H-mesulergine labeled native and mutant 5-HT_{2C} receptors. 0.5nM ^{3}H-mesulergine was used to label the native and mutant receptors transiently transfected in COS-7 cells. As shown in Figure 12, the 5-HT competition isotherms for ^{3}H-mesulergine labeled ser → lys and ser → phe mutant receptors display a marked leftward shift compared with native receptors. The affinity of 5-HT for ser → lys mutant receptors increased

almost 30-fold from 203 nM in the native to 6.6 nM in the ser → lys mutant. Similarly, but on a smaller scale, the ser → phe mutation resulted in a 3-fold increase in 5-HT affinity to 76 nM.

To determine whether other agonists would display a similar increase in affinity for the mutant receptors, two known agonists, 5-methoxytryptamine and DOB were tested with the ser → lys mutant. Figure 13 shows the radioligand binding analysis of native and mutant 5-HT_{2C} receptors in the presence of agonists and antagonists. Native and mutant 5-HT_{2C} receptors expressed in COS-7 cells were labeled with 1 nM 3H-mesulergine. The 5-MT and DOB agonists show increased affinity for the mutant receptor, as is seen for 5-HT. 5-methoxy-tryptamine and DOB display an 89-fold and 38-fold increase, respectively, in affinity for the ser → lys mutant receptors.

To determine if the mutant 5-HT_{2C} receptors would exhibit an increase in agonist potency relative to the native 5-HT_{2C} receptor, 5-HT stimulation of the native and mutant 5-HT_{2C} receptors was measured using an IP production assay. Figure 14 shows the stimulation of IP production in COS-7 cells expressing native or mutant 5-HT_{2C} receptors. Both the ser → lys and ser → phe mutant receptor curves exhibit a leftward shift away from the native curve in the 5-HT dose-response indicating that there was an increase in 5-HT potency for the mutant receptors. The shifts were similar in magnitude to the shifts in the 5-HT competition binding isotherms. Figure 15 shows the 5-HT stimulation of IP production in COS-7 cells transfected with the ser → lys or ser → phe mutated receptors. As shown in Figure 15, the EC_{50} value for 5-HT mediated stimulation of IP production increased from 70 nM in cells transfected with native receptors to 2.7 nM in the ser → lys mutant and 28 nM in the ser → phe mutant.

Figure 16 shows the effect of the ser → lys and ser → phe mutations on basal levels of IP production by the mutated 5-HT_{2C} receptors. Cells transfected with native 5-HT_{2C} receptors displayed a small increase (9%, 225dpm) in basal IP production over cells transfected with vector alone. Transfection with ser → lys and ser → phe mutant 5-HT_{2C} receptors resulted in 5-fold and 2-fold increases, respectively, in basal levels of IP production when compared with cells expressing native 5-HT_{2C} receptors. Basal levels of IP stimulated by ser → lys mutant

receptors represented 50% of total IP production stimulated by native receptors in the presence of 10 μM 5-HT. 5-HT stimulated IP production 10 fold over basal levels in cells transfected with native receptors and 2-fold over basal levels in cells transfected with ser → lys mutant receptors. However, 5-HT elicited the same maximal IP response in cells transfected with native or mutant receptors.

Since receptor density can influence agonist binding affinity and potency in stimulating second messenger systems, saturation curves were generated. Therefore, ^{3}H-mesulergine saturation analyses and Scatchard transformations were performed in parallel to control for variations in transfection efficiency and receptor expression levels. As shown in Figure 15, the 5-HT_{2C} receptor density was greater in cells transfected with native receptors than in cells transfected with either the ser → lys or the ser → phe mutant receptors. These data indicate that the increase in agonist binding affinity and potency of the mutated receptors did not result from increased receptor expression, but directly resulted from the mutations.

Thus, like the mutated 5-HT_{2A} receptors, the mutated 5-HT_{2C} receptors meet all the criteria for constitutively activated receptors; they show a higher affinity for agonists; they show a higher potency for 5-HT; and they show activation (coupling) of the G protein second messenger pathway (IP production) even in the absence of agonist.

Inverse Agonism at Constitutively Activated Serotonin Receptors

As noted above, the discovery and elucidation of the mechanisms of action of constitutively activated receptors has led to the recognition of a new class of receptor antagonists, identified as inverse agonists. The mutated 5-HT_{2A} and 5-HT_{2C} receptors of this invention were used to test the activity of known serotonin receptor antagonists. Figure 8 shows the binding affinities of four known 5-HT_{2A} antagonists to the native and cys → lys mutant 5-HT_{2A} receptors. There is an apparent decease in the binding affinity of methysergide and mianserin at the mutant 5-HT_{2A} receptors, but no change in binding affinity for spiperone and ketanserin. However, as shown in Figure 17, both spiperone and ketanserin reversed the constitutive stimulation of IP production in cells expressing the mutant 5-HT_{2A} receptor. Ketanserin and spiperone decreased the constitutive IP

stimulation by 80% and 58% respectively.

Several antipsychotic drugs presently in use are thought to act at the 5-HT_{2A} receptor. As shown in Figure 18, all these drugs, chlorpromazine, haloperidol, loxapine, clozapine, and risperidone as well as spiperone reduce the constitutively activated IP basal activity of the mutated 5-HT_{2A} receptor.

The constitutively active ser → lys mutated 5-HT_{2C} receptor of this invention can also be used to screen compounds for inverse agonist activity. Figure 19 shows that two classical 5-HT_{2C} receptor antagonists, mianserin and mesulergine, exhibit inverse agonist activity by decreasing basal levels of PI hydrolysis associated with the constitutively active 5-HT_{2C} mutant receptor. The inverse agonism of these compounds is apparent both in the presence and absence of serotonin.

The demonstration of inverse agonism at the mutated 5-HT_{2A} and 5-HT_{2C} receptors further characterizes the mutated serotonin receptors of this invention as being constitutively active. Not only have the 5-HT_{2A} and 5-HT_{2C} receptors been mutated to a constitutively active form, but a method has been disclosed for mutating all mammalian G protein-coupled monoamine receptors, including serotonin receptors, to a constitutively active form. Unlike the case of the M5 muscarinic acetylcholine receptor where mutations in the third cytoplasmic loop do not produce constitutive activation, the present invention clearly demonstrates that mutations in the third cytoplasmic loop of G protein-coupled serotonin receptors may be used to induce constitutive activation. Previously, third intracellular loop mutations near the transmembrane region had only been found to produce constitutively active receptors of the adrenergic type. With the present discoveries, it is now recognized that the alignment and positional mutation method of this invention is applicable to the general class of monoamine receptors of which the adrenergic and serotonin receptors are major subclasses. Further, based upon the present discoveries, it is expected that mutations may be introduced at other sites in the third cytoplasmic loop which will constitutively activate the G protein-coupled monoamine receptors including the serotonin receptors.

Additional Advances Enabled By The Discoveries Of The Present Invention:

Figures 20A and 20B show the DNA and amino acid sequences for the human 5-HT_{2A} receptors. In Figure 20A, it can be seen that the sixth transmembrane domain has the same WxPFFI conserved sequence (outlined type) as seen in the rat receptors. Figures 21A and 21B show the DNA and amino acid sequences for the human 5-HT_{2C} receptors. In Figure 21A it can be seen that the sixth transmembrane domain also has the same WxPFFI conserved sequence (outlined type) as seen in the rat receptors. Both of these human receptors may, therefore, be similarly aligned with the rat α1-adrenergic, 5-HT_{2A}, and 5-HT_{2C} receptors to identify the amino acid positions which may be mutated to produce constitutively active human receptors following the methodologies of this invention.

Having identified mutations which constitutively activate the 5-HT_{2A} and 5-HT_{2C} serotonin receptors, it is now possible to create transgenic mammals incorporating these mutations using techniques well known in the art. This will provide an opportunity to study the physiological consequences of constitutive receptor activation and may lead to the development of novel therapeutic agents.

Those skilled in the art will recognize that various modifications, additions, substitutions and variations of the illustrative examples set forth herein can be made without departing from the spirit of the invention and are, therefore, considered within the scope of the invention.

References

1. Cotecchia, S, Exum, S., Caron, M., and Lefkowitz, R. (1990) *Regions of the α_1-adrenergic receptor involved in coupling to phosphatidylinositol hydrolysis and enhanced sensitivity of biological function*. Proc. Natl. Acad. Sci. Vol 87, 2896-2900

2. Kjelsberg, M., Cotecchia, S., Ostrowski, J. Caron, M. and Lefkowitz, R. (1992) *Constitutive Activation of the α_{1B}-Adrenergic Receptor by All Amino Acid Substitutions at a Single Site*. J. Biol. Chem. Vol. 267, 1430-1433

3. Samama, P., Cotecchia, S., Costa, T. and Lefkowitz, R. (1993) *A Mutation-induced Activated State of the B_2-Adrenergic Receptor*. J. Biol.

Chem. Vol 268, 4625-4636

4. Burstein, E., Spalding, T., Hill-Eubanks, D., & Brann, M. (1995) *Structure-Function of Muscarinic Receptor Coupling to G Proteins*. J. Biol. Chem. Vol 270, 3141-3146

5. The native rat 5-HT_{2A} receptor cDNA was generously donated by Dr. David Julius of the University of California, San Francisco.

6. The native rat $5HT_{2C}$ receptor cDNA was generously donated by Dr. Beth Hoffman of the National Institutes of Health.

CLAIMS

What is claimed is:

1. A method of constitutively activating targeted G protein-coupled mammalian monoamine receptors comprising the following steps:

a. aligning a conserved amino acid sequence occurring in the sixth transmembrane domain of the targeted monoamine receptor with the conserved amino acid sequence in the sixth transmembrane domain of a second monoamine receptor for which a constitutively activated form having a mutation in the third intracellular loop is known;

b. identifying in the aligned receptor sequences the amino acid position in the targeted monoamine receptor which corresponds to the amino acid position in the third intracellular loop which produced constitutive activation in the second monoamine receptor; and

c. mutating, by site-directed mutagenesis, the identified amino acid position in the targeted monoamine receptor so that a different amino acid is substituted for the amino acid occurring in the native targeted receptor.

2. The method of claim 1 in which the targeted monoamine receptor is a G protein-coupled serotonin receptor.

3. The method of claim 2 in which the G protein-coupled serotonin receptor is the 5-HT_{2A} receptor.

4. The method of claim 2 in which the G protein-coupled serotonin receptor is the 5-HT_{2C} receptor.

5. The method of claim 1 in which the conserved amino acid sequence within the sixth transmembrane domain used for the alignment is WxPFFI, where x represents that any amino acid may occur at that position.

6. A method of constitutively activating G protein-coupled mammalian serotonin receptors comprising the following steps:

a. aligning a conserved amino acid sequence occurring in the sixth transmembrane domain of the serotonin receptor with the conserv d amino acid sequence in the sixth transmembrane domain of the α_{1B}-adrenergic receptor for which a constitutively activated form having

a mutation in the third intracellular loop is known;

b. identifying in the aligned receptor sequences the amino acid position in the serotonin receptor which corresponds to the amino acid position in the third intracellular loop which produced constitutive activation in the α_{1B}-adrenergic receptor; and

c. mutating, by site-directed mutagenesis, the identified amino acid position in the serotonin receptor so that a different amino acid is substituted for the amino acid occurring in the native serotonin receptor.

7. The method of claim 6 in which the G protein-coupled serotonin receptor is the 5-HT_{2A} receptor.

8. The method of claim 6 in which the G protein-coupled serotonin receptor is the 5-HT_{2C} receptor.

9. The method of claim 6 in which the conserved amino acid sequence within the sixth transmembrane domain used for the alignment is WxPFFI, where x represents that any amino acid may occur at that position.

10. The constitutively active 5-HT_{2A} receptor in which the amino acid at position number 322 has been mutated from the cysteine found in the native receptor to an amino acid selected from the group consisting of lysine, glutamic acid, and arginine.

11. The constitutively active 5-HT_{2C} receptor in which the amino acid at position number 312 has been mutated from the serine found in the native receptor to an amino acid selected from the group consisting of lysine and phenylalanine.

12. The DNA encoding the constitutively active 5-HT_{2A} receptor in which the amino acid at position number 322 has been mutated from the cysteine found in the native receptor to an amino acid selected from the group consisting of lysine, glutamic acid, and arginine.

13. The DNA encoding the constitutively active 5-HT_{2C} receptor in which the amino acid at position number 312 has been mutated from the serine found in the native receptor to an amino acid selected from the group consisting of lysine and phenylalanine.

14. A method of efficiently minimizing the number of full DNA sequencings, which must be performed on the colonies resulting from site-directed mutagenesis employing vectors, by eliminating most colonies not containing the desired mutation and by tagging colonies containing the desired mutation for easy identification comprising the following steps:

a. creating two primers, the first of which will remove a restriction site occurring in the original form of the vector and the second of which will introduce the desired mutation as well as a second mutation which specifies a unique restriction site not found in the primer;

b. annealing the primers to the vector;

c. synthesizing the second strands;

d. exposing the double stranded DNA to the restriction enzyme for the restriction site which occurs on the original vector thereby digesting the DNA containing the restriction site so that it cannot be taken up during a subsequent transformation;

e. transforming the test organism with the remaining double stranded circular DNA; and

f. testing the resulting colonies to see if they contain DNA which can be digested by the restriction enzyme for the unique site introduced by the second primer

whereby only DNA from those colonies which have incorporated the desired mutation will be digested with the restriction enzyme for the unique restriction site and the presence of such digestion indicates that that colony contains the desired mutation.

15. The method of claim 14 in which the following additional steps are performed after step e and before step f of claim 14:

e'. repeating a restriction digest using the restriction enzyme for the restriction site which occurs on the original vector; and

e''. tansforming the test organism with the remaing double stranded circular DNA.

16. The constitutively active 5-HT_{2A} receptor coded by the DNA sequence specified in Figure 24 which DNA also contains a mutation creating a unique

restriction site.

17. The c nstitutively active 5-HT_{2A} receptor coded by the DNA sequence specified in Figure 27 which DNA also contains a mutation creating a unique restriction site.

18. The constitutively active 5-HT_{2A} receptor coded by the DNA sequence specified in Figure 30 which DNA also contains a mutation creating a unique restriction site.

19. The constitutively active 5-HT_{2C} receptor coded by the DNA sequence specified in Figure 33 which DNA also contains a mutation creating a unique restriction site.

20. The constitutively active 5-HT_{2C} receptor coded by the DNA sequence specified in Figure 36 which DNA also contains a mutation creating a unique restriction site.

21. The use of the constitutively activated mammalian G protein-coupled monamine receptor to screen for agonists, inverse agonists, and antagonists not previously identified as such at the native receptor.

22. The method of claim 21 where the mammalian G protein-coupled monoamine receptor is a serotonin receptor.

23. A transgenic mammal having incorporated and expressed in its genome a constitutively activated monoamine G protein-coupled receptor.

24. The transgenic mammal of claim 23 wherein the constitutively activated monoamine G protein-coupled receptor is a serotonin receptor.

25. The method of constitutively activating G protein-coupled receptors as described and illustrated in the specification.

26. The method of efficiently minimizing the number of full DNA sequencings as described and illustrated in the specification.

27. The constitutively activated receptors as described and illustrated in the specification.

28. DNA encoding constitutively activated receptors as described and illustrated in the specification.

29. The invention as described and illustrated in the specification.

Rat 5-HT_{2A}

1 cccaggctat gaacccctag tctctccaca cttcatctgc tacaacttcc ggcttagaca

61 tggaaattct ttgtgaagac aatatctctc tgagctcaat tccaaactcc ttaatgcaat

121 taggtgatgg cccgaggctc taccataatg acttcaactc cagagatgct aacacttcgg

181 aagcatcgaa ctggacaatt gatgctgaaa acagaaccaa cctctcctgt gaagggtacc

241 tcccaccgac atgcctctcc attcttcatc tccaggaaaa aaactggtct gctttattga

301 caactgtcgt gattattctc accattgctg gaaatatact ggtcatcatg gcagtgtccc

361 tagaaaaaaa gctgcagaat gccaccaact atttcctgat gtcacttgcc atagctgata

421 tgctgctggg tttccttgtc atgcctgtgt ccatgttaac catcctgtat gggtaccggt

481 ggcctttgcc tagcaagctc tgtgcgatct ggatttacct ggatgtgctc ttttctacgg

541 catccatcat gcacctctgc gccatctccc tggaccgcta tgtcgccatc cagaacccca

601 ttcaccacag ccgcttcaac tccagaacca aagccttcct gaaaatcatt gccgtgtgga

661 ccatatctgt aggtatatcc atgccaatcc cagtctttgg actacaggat gattcgaagg

721 tctttaagga ggggagctgc ctgcttgccg atgacaactt tgttctcata ggctcttttg

781 tggcattttt catcccccta accatcatgg tgatcaccta cttcctgact atcaagtcac

841 ttcagaaaga agccaccttg tgtgtgagtg acctcagcac tcgagccaaa ctagcctcct

901 tcagcttcct ccctcagagt tctctgtcat cagaaaagct cttccaacgg tccatccaca

961 gagagccagg ctcctacgca ggccgaagga cgatgcagtc catcagcaat gagcaaaagg

1021 cgtgcaaggt gctgggcatc gtgttcttcc tgtttgttgt aatgtggtgc ccattcttca

1081 tcaccaatat catggccgtc atctgcaaag aatcctgcaa tgaaaatgtc atcggagccc

1141 tgctcaatgt gtttgtctgg attggttatc tctcctcagc tgtcaatcca ctggtatata

1201 cgttgttcaa taaaacttat aggtccgcct tctcaaggta cattcagtgt cagtacaagg

1261 aaaacagaaa gccactgcag ttaattttag tgaacactat accagcattg gcctacaagt

FIGURE 1A

1321 ctagtcagct ccaggtggga cagaaaaaga actcacagga agatgctgag cagacagttg

1381 atgactgctc catggttaca ctggggaaac aacagtcgga agaggaattgt acaggacaata

1441 ttgaaaccgt gaatgaaaag gttagctgtg tgtgatgaac tggatgctat ggcaattgcc

1501 cagggcatgt gaacaaggtt atacccatgt gtgtggggcg gggataagga ggctgcaaca

1561 aattag

FIGURE 1A - CONTINUED

Rat 5-HT_{2A}

MEILCEDNISLSSIPNSLMQLGDGPRLYHNDFNSRDANTSEASN

WTIDAENRTNLSCEGYLPPTCLSILHLQEKNWSALLTTVVIILTIAGNILVIMAVSLE

KKLQNATNYFLMSLAIADMLLGFLVMPVSMLTILYGYRWPLPSKLCAIWIYLDVLFST

ASIMHLCAISLDRYVAIQNPIHHSRFNSRTKAFLKIIAVWTISVGISMPIPVFGLQDD

SKVFKEGSCLLADDNFVLIGSFVAFFIPLTIMVITYFLTIKSLQKEATLCVSDLSTRA

KLASFSFLPQSSLSSEKLFQRSIHREPGSYAGRRTMQSISNEQKACKVLGIVFFLFVV

MWCPFFITNIMAVICKESCNENVIGALLNVFVWIGYLSSAVNPLVYTLFNKTYRSAFS

RYIQCQYKENRKPLQLILVNTIPALAYKSSQLQVGQKKNSQEDAEQTVDDCSMVTLGK

QQSEENCTDNIETVNEKVSCV

FIGURE 1B

Rat 5-HT_{2c}

ORIGIN 23 bp upstream of HindIII site.

1 ggcgctctgg tgctcactga ggaagcttcc ttaggtgtac cgatcttaat gattgagccc

61 ttggagcagc aagattgtta atcttggttg ctcctttggc ctgtctatcc cttaccttcc

121 tattacatat gaacttttct tcgttctgca catcgattgt cgtcggcgtc gtggagatcg

181 tcgtggtgct ccggtggtgg tcttcgtccg cttagaatag tgtagttagt taggggcctt

241 caaagaagaa agaagaagcg attggcgcgg agagatgctg gaggtgtcag tttctatgct

301 agagtagggt agtgaaacaa tccccagcca aacctttccg gggggcgcag gttgcccaca

361 ggaggtcgac ttgccggcgc tgtccttcgc gccgagctcc ctccatcctt ctttccgtct

421 gctgagacgc aaggttgcgg cgcgcacgct gagcagcgca ctgactgccg cgggctccgc

481 tgggcgattg cagccgagtc cgtttctcgt ctagctgccg ccgcggcgac ctgcctggtc

541 ttcctcccgg acgctagcgg gttgtcaact attacctgca agcataggcc aacgaacacc

601 ttctttccaa attaattgga atgaaacaat tctgttaact tcctaattct cagtttgaaa

661 ctctggttgc ttaagcctga agcaatcatg gtgaaccttg gcaacgcggt gcgctcgctc

721 ctgatgcacc taatcggcct attggtttgg caattcgata tttccataag tccagtagca

781 gctatagtaa ctgacacttt taattcctcc gatggtggac gcttgtttca attcccggac

841 ggggtacaaa actggccagc actttcaatc gtcgtgatta taatcatgac aatagggggc

901 aacattctta ttatcatggc agtaagcatg gagaagaaac tgcacaatgc aaccaattac

961 ttcttaatgt ccctagccat tgctgatatg ctggtgggac tacttgtcat gcccctgtcc

1021 ctgcttgcta ttctttatga ttatgtctgg cctttaccta gatatttgtg ccccgtctgg

1081 atttcactag atgtgctatt ttcaactgcg tccatcatgc acctctgcgc catatcgctg

1141 gaccggtatg tagcaatacg taatcctatt gagcatagcc ggttcaattc gcggactaag

1201 gccatcatga agattgccat cgtttgggca atatcaatag gagtttcagt tcctatccct

FIGURE 2A

1261 gtgattggac tgagggacga aagcaaagtg ttcgtgaata acaccacgtg cgtgctcaat

1321 gaccccaact tcgttctcat cgggtccttc gtggcattct tcatcccgtt gacgattatg

1381 gtgatcacct acttcttaac gatctacgtc ctgcgccgtc aaactctgat gttacttcga

1441 ggtcacaccg aggaggaact ggctaatatg agcctgaact ttctgaactg ctgctgcaag

1501 aagaatggtg gtgaggaaga gaacgctccg aaccctaatc cagatcagaa accacgtcga

1561 aagaagaaag aaaagcgtcc cagaggcacc atgcaagcta tcaacaacga aaagaaagct

1621 tccaaagtcc ttggcattgt attctttgtg tttctgatca tgtggtgccc gtttttcatc

1681 accaatatcc tgtcggttct ttgtgggaag gcctgtaacc aaaagctaat ggagaagctt

1741 ctcaatgtgt ttgtgtggat tggctatgtg tgttcaggca tcaatcctct ggtgtacact

1801 ctctttaata aaatttaccg aagggctttc tctaaatatt tgcgctgcga ttataagcca

1861 gacaaaaagc ctcctgttcg acagattcct agggttgctg ccactgcttt gtctgggagg

1921 gagctcaatg ttaacattta tcggcatacc aatgaacgtg tggctaggaa agctaatgac

1981 cctgagcctg gcatagagat gcaggtggag aacttagagc tgccagtcaa cccctctaat

2041 gtggtcagcg agaggattag tagtgtgtaa gcgaagagca gcgcagactt cctacaggaa

2101 agttcctgta ggaaagtcct ccccaccccc cgtgattttc ctgtgaatca taactaatgt

2161 aaatattgct gtgtgacaag acagtgtttt tataaatagc tttgcaaccc tgtactttac

2221 atcatgcgtt aatagtgaga ttcggg

FIGURE 2A - CONTINUED

Rat 5-HT_{2C}

MVNLGNAVRSLLMHLIGLLVWQFDISISPVAAIVTDTFNSSDGG

RLFQFPDGVQNWPALSIVVIIIMTIGGNILVIMAVSMEKKLHNATNYFLMSLAIADML

VGLLVMPLSLLAILYDYVWPLPRYLCPVWISLDVLFSTASIMHLCAISLDRYVAIRNP

IEHSRFNSRTKAIMKIAIVWAISIGVSVPIPVIGLRDESKVFVNNTTCVLNDPNFVLI

GSFVAFFIPLTIMVITYFLTIYVLRRQTLMLLRGHTEEELANMSLNFLNCCCKKNGGE

EENAPNPNPDQKPRRKKKEKRPRGTMQAINNEKKASKVLGIVFFVFLIMWCPFFITNI

LSVLCGKACNQKLMEKLLNVFVWIGYVCSGINPLVYTLFNKIYRRAFSKYLRCDYKPD

KKPPVRQIPRVAATALSGRELNVNIYRHTNERVARKANDPEPGIEMQVENLELPVNPS

NVVSERISSV

FIGURE 2B

Rat α_{1B}-adrenergic

MNPDLDTGHNTSAPAHWGELKDDNFTGPNQTSSNSTLPQLDVTR

AISVGLVLGAFILFAIVGNILVILSVACNRHLRTPTNYFIVNLAIADLLLSFTVLPFS

ATLEVLGYWVLLSFFCDIWAAVDVLCCTASILSLCAISIDRYIGVRYSLQYPTLVTRR

KAILALLSVWVLSTVISIGPLLGWKEPAPNDDKECGVTEEPFYALFSSLGSFYIPLAV

ILVMYCRVYIVAKRTTKNLEAGVMKEMSNSKELTLRIHSKNFHEDTLSSTKAKGHNPR

SSIAVKLFKFSREKKAAKTLGIVVGMFILCWLPFFIALPLGSLFSTLKPPDAVFKVVF

WLGYFNSCLNPIIYPCSSKEFKRAFMRILGCQCRGGRRRRRRRRLGACAYTYRPWTRG

GSLERSQSRKDSLDDSGSCMSGTQRTLPSASPSPGYLGRGTQPPVELCAFPEWKPGAL

LSLPEPPGRRGRLDSGPLFTFKLLGDPESPGTEGDTSNGGCDTTTDLANGQPGFKSNM

PLAPGHF

FIGURE 3A

Rat α_{1B}-adrenergic

1 gggcggactt taaaatgaat cccgatctgg acaccggcca caacacatca gcacctgccc

61 actggggaga gttgaaagat gacaacttca ctggccccaa ccagacctcg agcaactcca

121 cactgcccca gctggacgtc accagggcca tctctgtggg cctggtgctg ggcgccttca

181 tcctctttgc catcgtgggc aacatcttgg tcatcctgtc ggtggcctgc aaccggcacc

241 tgcggacgcc caccaactac tttatcgtca acctggccat tgctgacctg ctgttgagtt

301 tcacagtact gcccttctcc gctaccctag aagtgcttgg ctactgggtg ctgttgagtt

361 tcttctgtga catctgggca gcggtagatg tcctgtgctg tacggcctcc atcctgagcc

421 tatgtgccat ctccattgac cgctacattg gggtgcgata ctctctgcag taccccacgc

481 tggtcacccg caggaaggcc atcttggcgc tcctcagtgt gtgggtcttg tccacggtca

541 tctccatcgg gcctctcctt ggatggaaag aacctgcgcc caatgatgac aaagaatgtg

601 gggtcaccga agaacccttc tacgccctct tttcctccct gggctccttc tacatcccgc

661 tcgcggtcat cctggtcatg tactgccggg tctacatcgt ggccaagagg accaccaaga

721 atctggaggc gggagtcatg aaggaaatgt ccaactccaa ggagctgacc ctgaggatcc

781 actccaagaa ctttcatgag gacaccctca gcagtaccaa ggccaagggc cacaacccca

841 ggagttccat agctgtcaaa cttttaagt tctccaggga aaagaaagca gccaaaacct

901 tgggcattgt agtcggaatg ttcatcttat gttggctccc cttcttcatc gctctcccgc

961 ttggctccct gttctccacc ctaaagcccc cggacgccgt gttcaaggtg gtgttctggc

1021 tgggctactt caacagctgc ctcaatccca tcatctaccc gtgctccagc aaggagttca

1081 agcgcgcctt catgcgtatc cttgggtgcc agtgccgcgg tggccgccgc cgccgccgcc

1141 gtcgccgtct aggcgcgtgc gcttacacct accggccgtg gacccgcggc ggctcgctgg

1201 agagatcaca gtcgcggaag gactctctgg atgacagcgg caggtgcatg agcggcacgc

1261 agaggaccct gccctcggcg tcgcccagcc cgggctacct gggtcgagga acgcagccac

FIGURE 3B

1321 ccgtggaggt gtgcgccttc cccgagtgga aacccggggc gctgctcagc ttgccagagc

1381 ctcctggccg ccgcggccgt ctcgactctg ggccactctt caccttcaag ctcctgggcg

1441 atcctgagag cccgggaacc gaaggcgaca ccagcaacgg gggctgcgac accacgaccg

1501 acctggccaa cgggcagccc ggcttcaaga gcaacatgcc cctggcgccc gggcacttt

1561 agggtccctt ttcatcctcc ccctcaacac actcacacat cggggtgggg gagaacacca

1621 tcgtaggggc gggagggcgc gtgggggggag tgtcagccct aggtagacac agggtcgcaa

1681 ggggacaagg ggggaggggg gcggggagag gggcagctgc ttttctggca ggggcatggg

1741 tgccaggtac agcgaagagc tgggctgagc atgctgagag cgtgggggggc ccccctagtg

1801 gttccgggac ttaagtctct ctctcttctc tctctgtata tacataaaat gagttcctct

1861 attcgtattt atctgtgggt acacgtgcgt gtgtctgttc ggtgtacgtg tgggctgcat

1921 gggtgtgagt gtgaggcctg cccgcacgcg cgtgccgggg cagagcgagt gcgcccccctg

1981 gtgacgtcca ggtgtgttgt ttgtctcttg actttgtacc tctcaagccc ctccctgttc

2041 tctagtcaat gctggcactt tgataggatc ggaaaacaag tcagatatta aagatcattt

2101 ctcctgtg

FIGURE 3B - CONTINUED

10/58

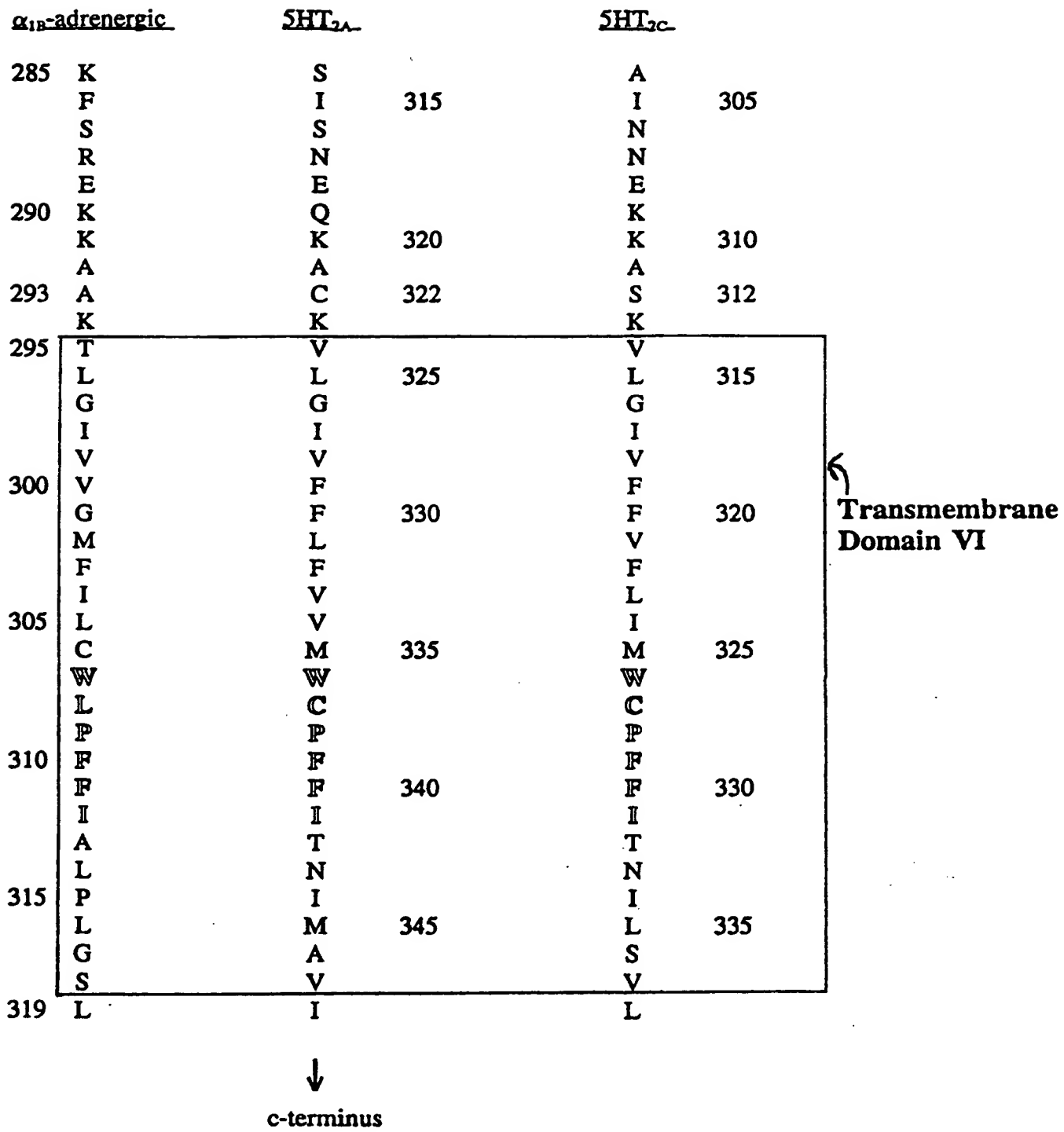


FIGURE 4

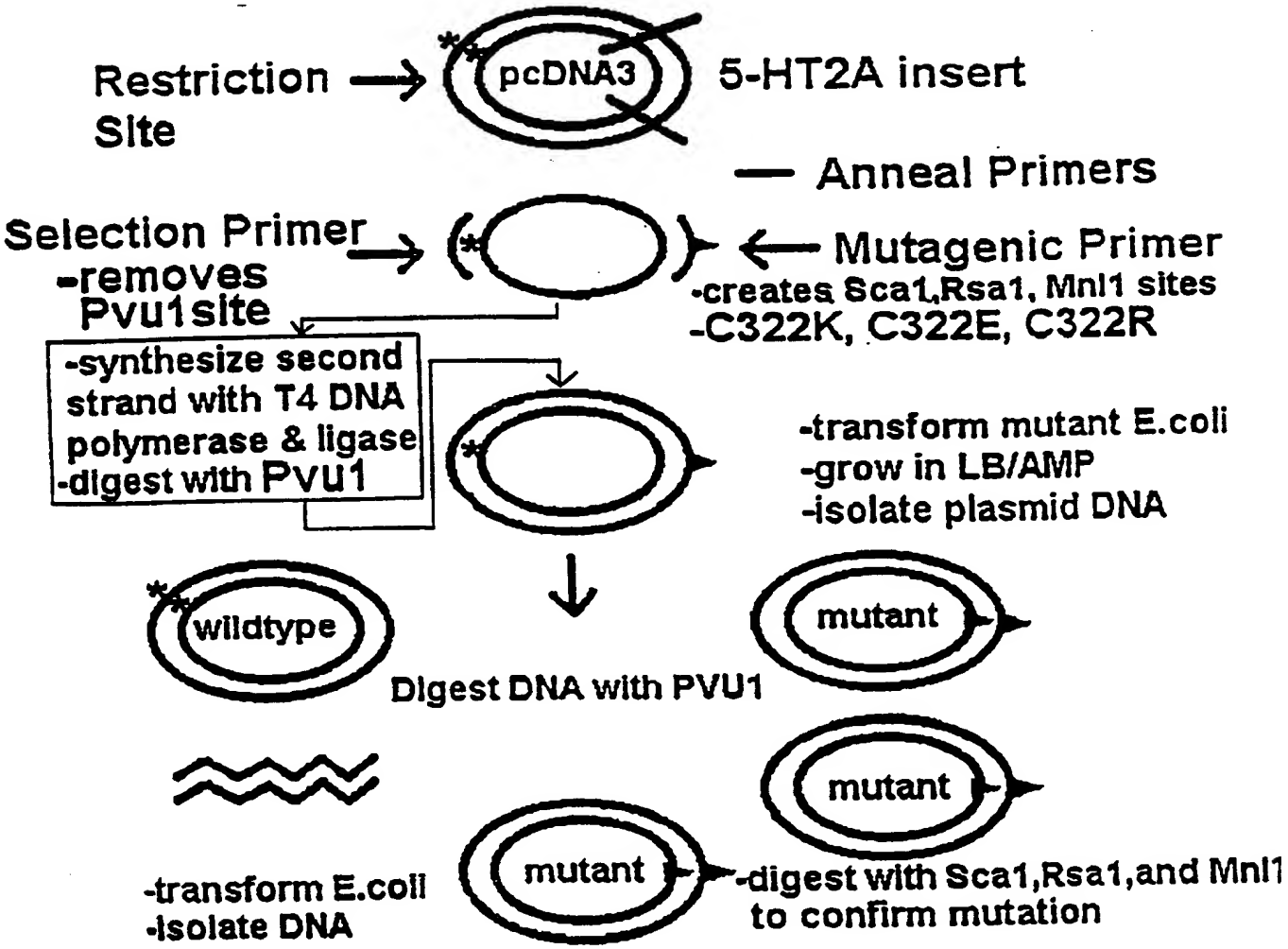

Restriction Site
pcDNA3
5-HT2A insert
Anneal Primers
Selection Primer
-removes Pvu1site
Mutagenic Primer
-creates Sca1,Rsa1, Mnl1 sites
-C322K, C322E, C322R
-synthesize second strand with T4 DNA polymerase & ligase
-digest with Pvu1
-transform mutant E.coli
-grow in LB/AMP
-isolate plasmid DNA
wildtype
mutant
Digest DNA with PVU1
mutant
mutant
-transform E.coli
-isolate DNA
-digest with Sca1,Rsa1,and Mnl1 to confirm mutation

FIGURE 5

Site-directed mutagenesis procedure for the 5-HT2C receptor.

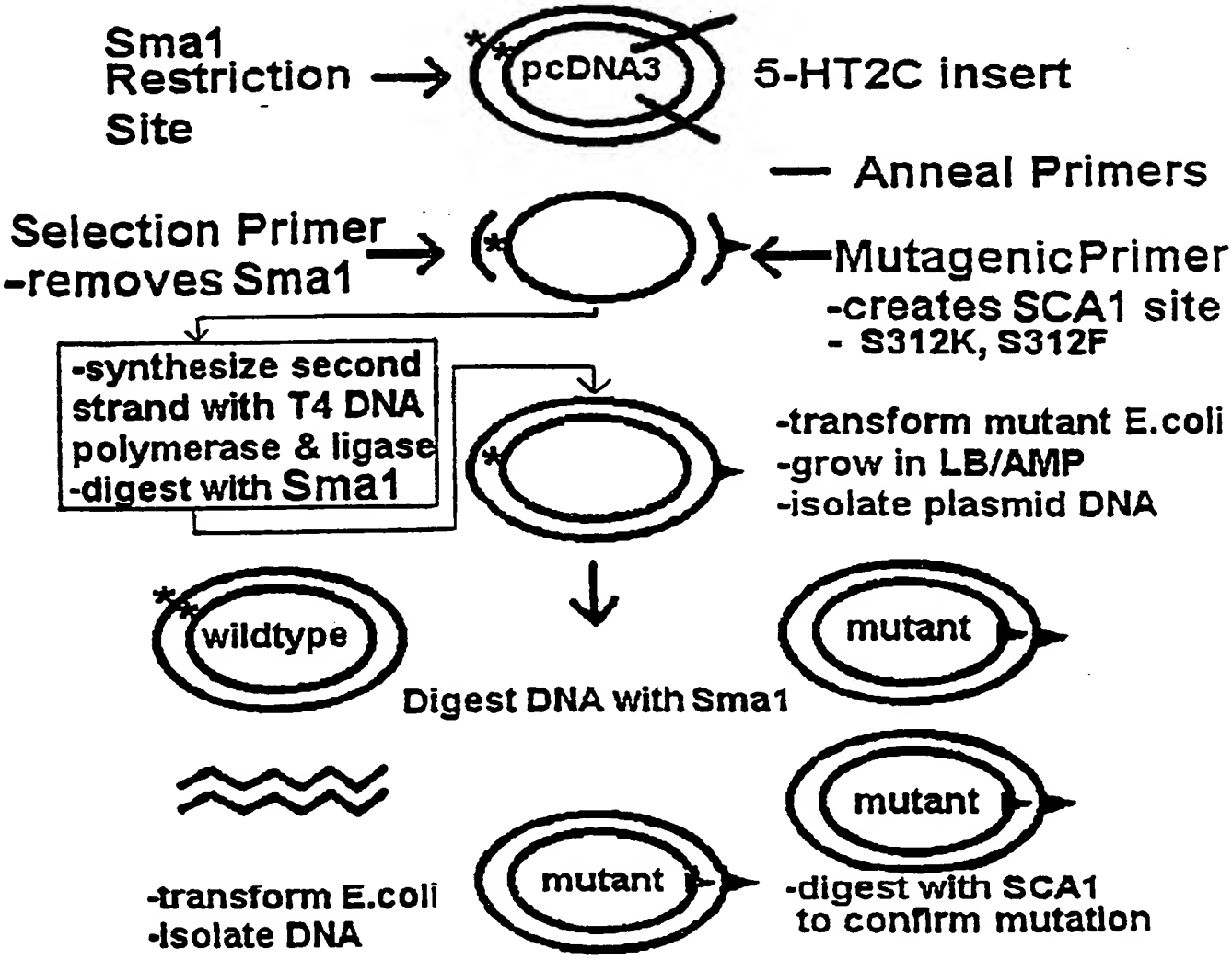


FIGURE 6

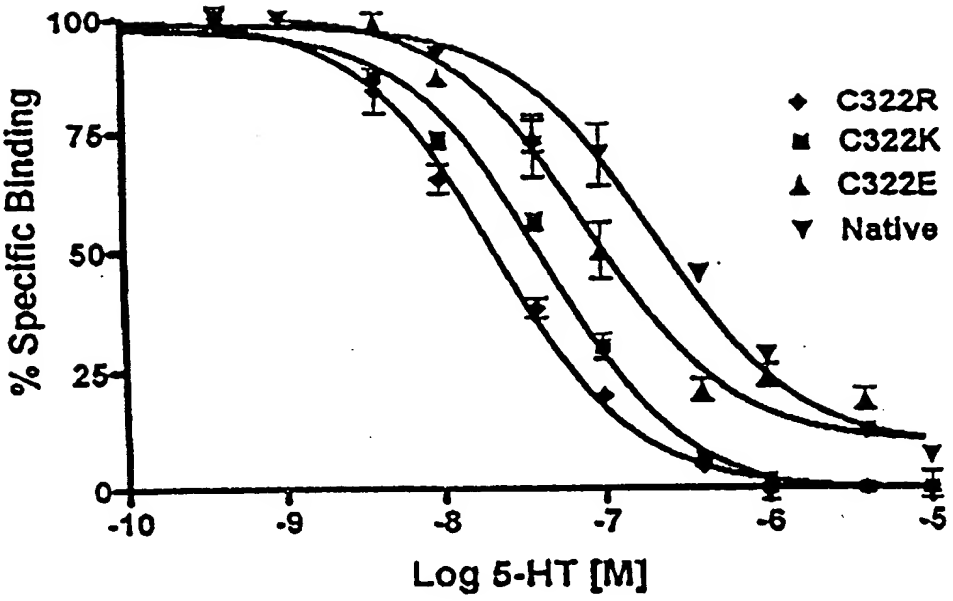

100
75
50
25
0
% Specific Binding
-10
-9
-8
-7
-6
-5
Log 5-HT [M]
C322R
C322K
C322E
Native

FIGURE 7

	Native 5-HT_{2A}	Cys→Lys Mutant	Cys→Arg Mutant	Cys→Glu Mutant
Agonists				
5-HT	293±3.0	25±2.1*	10±1.7	86±2.9
DOB	17±1.4	2.3±0.3*		
DOM	144±52	28±0.3*		
Antagonists				
Spiperone	1.1±0.1	2.4±1.0		
Methysergide	0.3±0.1	6.0±0.7*		
Ketanserin	1.0±0.3	1.0±0.1		
Mianserin	3.9±2.2	13±2.0*		

FIGURE 8

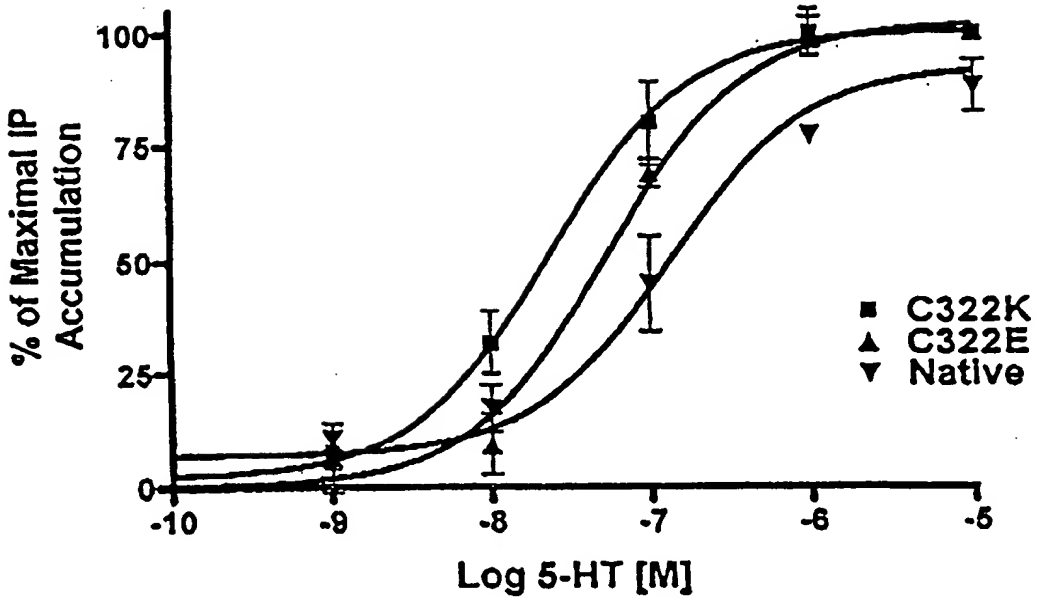

% of Maximal IP Accumulation
100
75
50
25
0
-10
-9
-8
-7
-6
-5
Log 5-HT [M]
C322K
C322E
Native

FIGURE 9

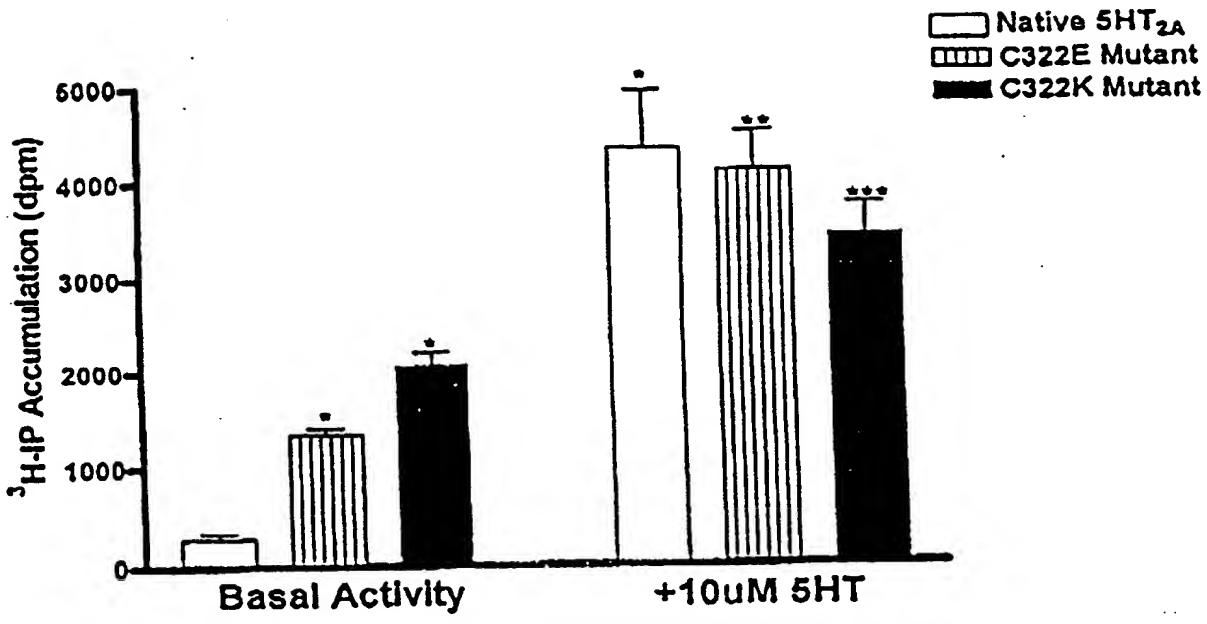
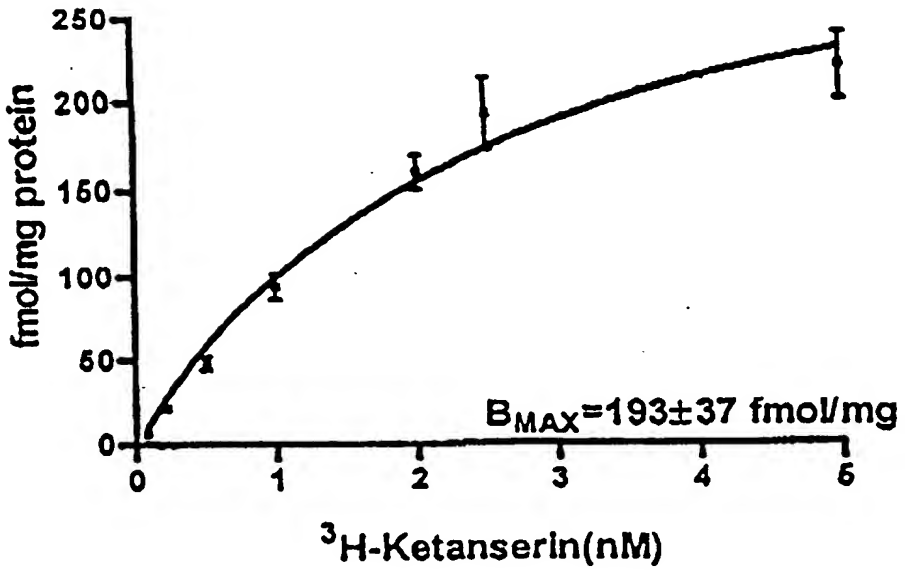

Native 5HT2A
C322E Mutant
C322K Mutant
3H-IP Accumulation (dpm)
6000
4000
3000
2000
1000
0
*
*
*
**
***
Basal Activity
+10uM 5HT

FIGURE 10


250
200
150
100
50
0
fmol/mg protein
B_{MAX}=193±37 fmol/mg
0
1
2
3
4
5
^{3}H-Ketanserin(nM)

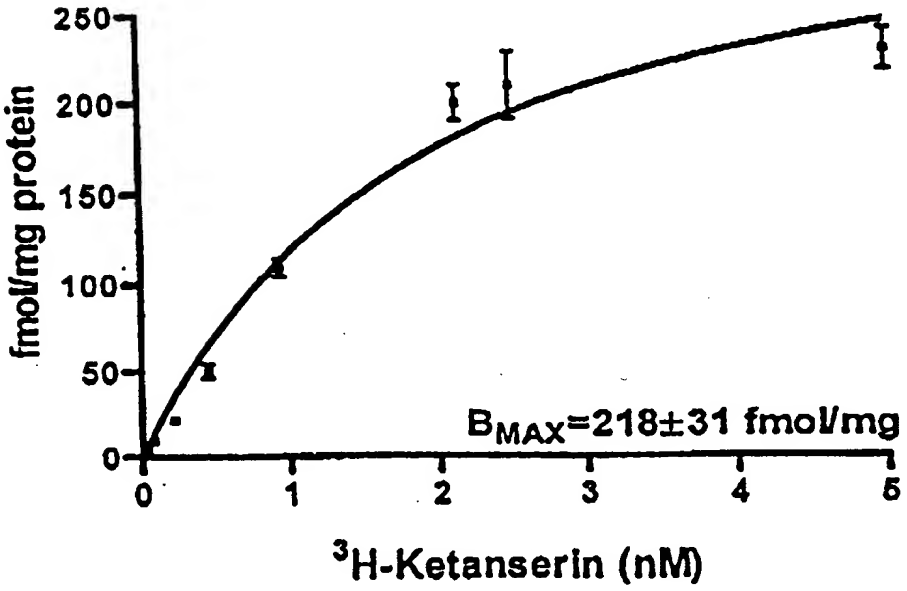

250
200
150
100
50
0
fmol/mg protein
B_{MAX}=218±31 fmol/mg
0
1
2
3
4
5
^{3}H-Ketanserin (nM)

FIGURE 11

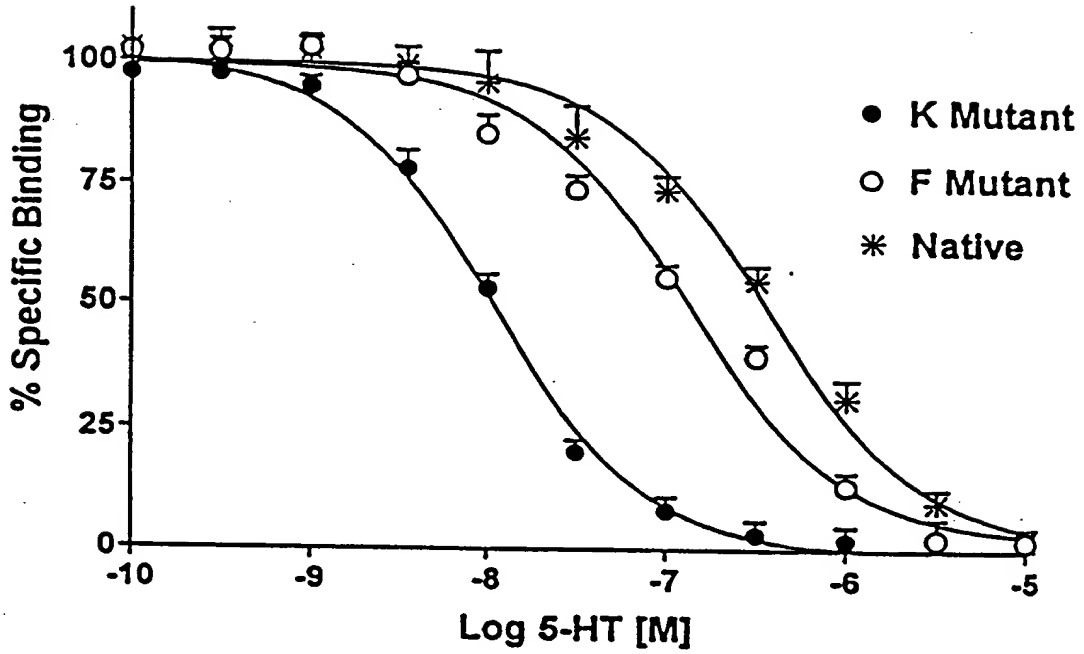

100
75
50
25
0
% Specific Binding
-10
-9
-8
-7
-6
-5
Log 5-HT [M]
K Mutant
F Mutant
Native

FIGURE 12

	Ki (nM)		
Agonists	Native	K Mutant	F Mutant
5-HT	203+/-10	6.6+/-1.2*	76+/-7.1*
5-MT	519+/-104	5.8+/-1.1*	ND
(+/-)DOB	256+/-38	6.7+/-0.7*	ND
Antagonists			
Mesulergine[a]	0.6+/-0.1	1.2+/-0.1*	1.3+/-0.2*
Mianserin	1.7+/-0.2	3.0+/-0.7**	ND
Methysergide	0.5+/-0.1	0.9+/-0.1**	ND

FIGURE 13

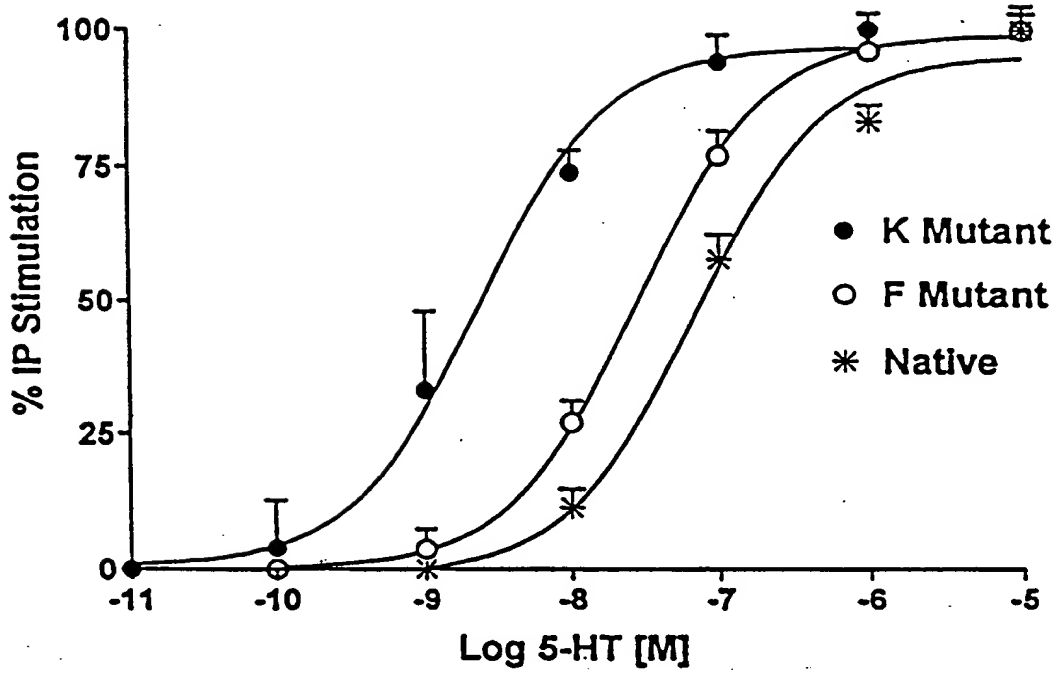

100
75
50
25
0
% IP Stimulation
-11
-10
-9
-8
-7
-6
-5
Log 5-HT [M]
K Mutant
F Mutant
Native

FIGURE 14

5-HT_{2c} Receptor	5-HT EC_{50} (nM)	K_D (nM)	Bmax (pm/mg)
Native	70+/-18	0.6+/-0.1	1.5+/-0.2
F Mutant	28+/-2.5*	1.3+/-0.2*	0.6+/-0.1*
K Mutant	2.7+/-1.1*	1.2+/-0.1*	1.4+/-0.2

FIGURE 15

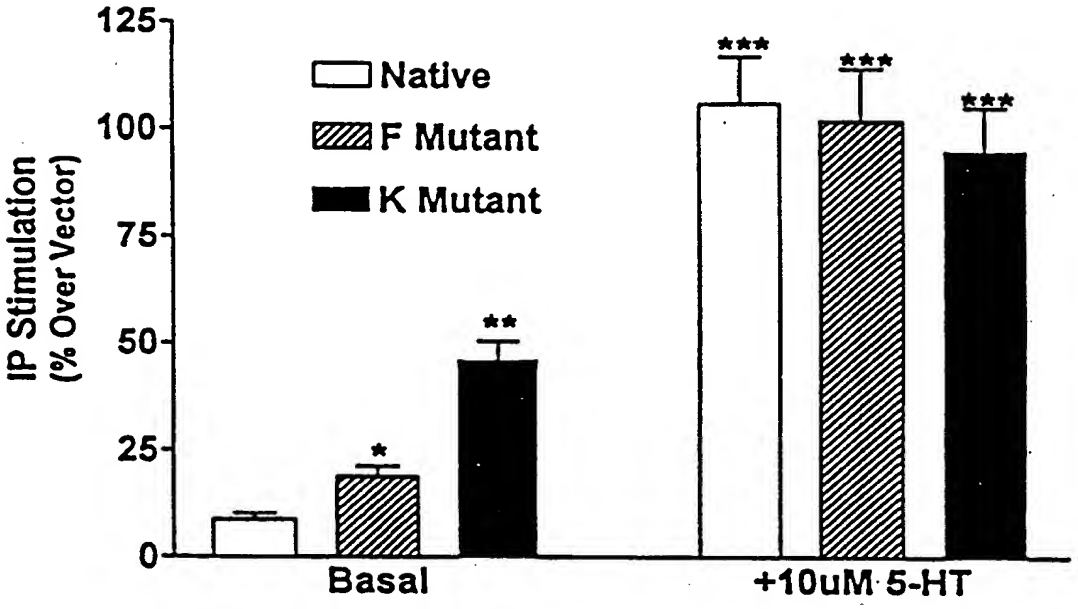

125
100
75
50
25
0
IP Stimulation
(% Over Vector)
Native
F Mutant
K Mutant
*
**
***
***
***
Basal
+10uM 5-HT

FIGURE 16

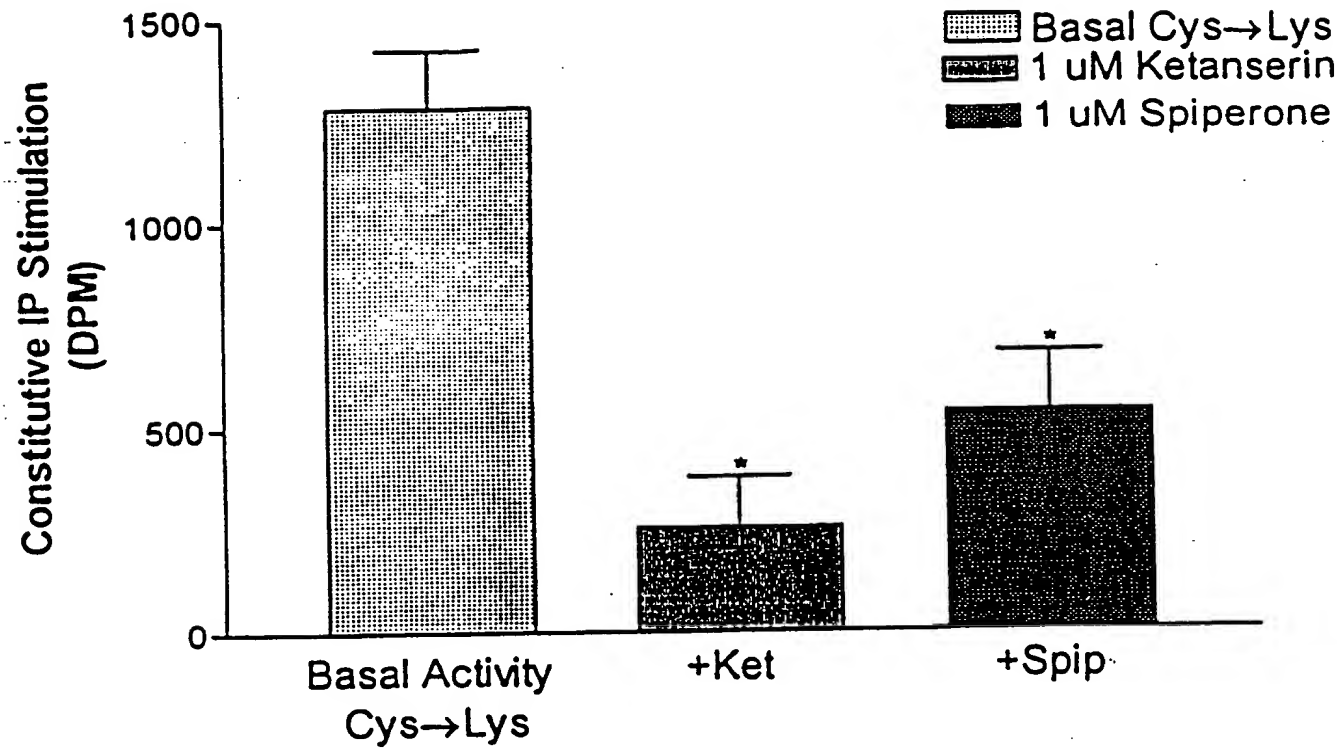

Basal Cys→Lys
1 uM Ketanserin
1 uM Spiperone
Constitutive IP Stimulation (DPM)
1500
1000
500
0
Basal Activity Cys→Lys
+Ket
+Spip

FIGURE 17

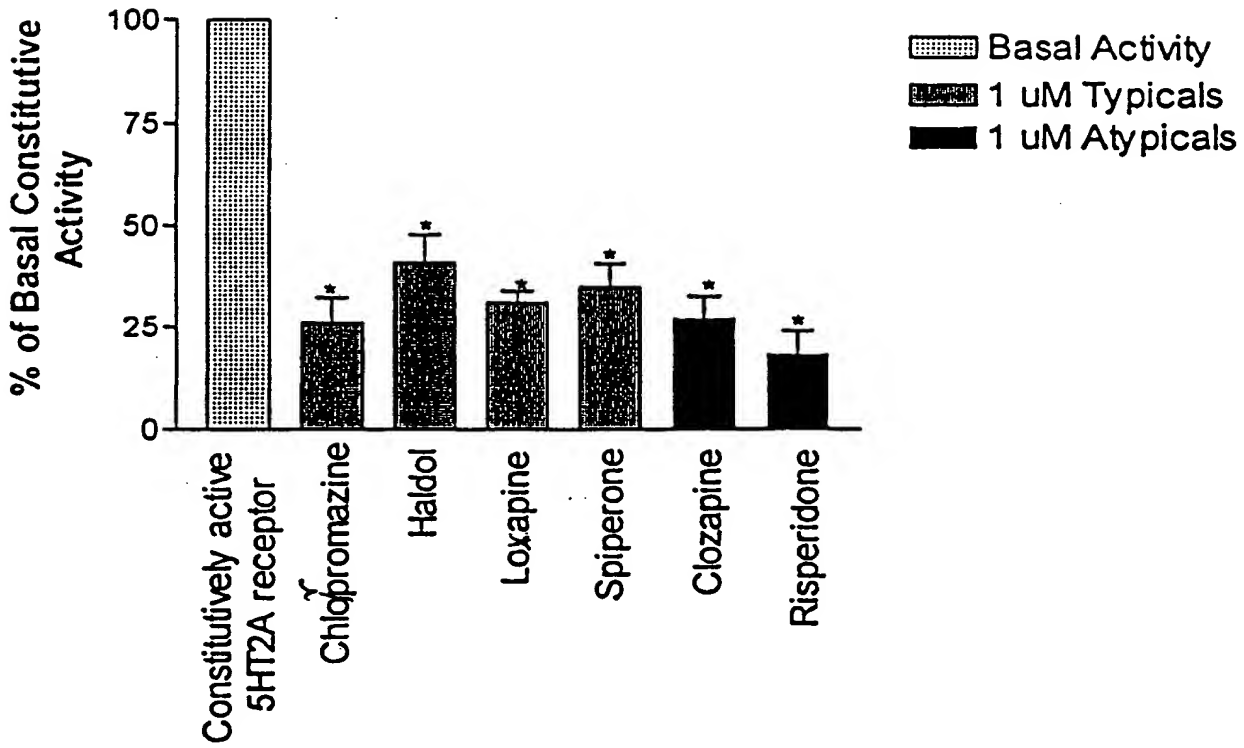

% of Basal Constitutive Activity
100
75
50
25
0
Basal Activity
1 uM Typicals
1 uM Atypicals
Constitutively active 5HT2A receptor
Chlorpromazine
Haldol
Loxapine
Spiperone
Clozapine
Risperidone

FIGURE 18

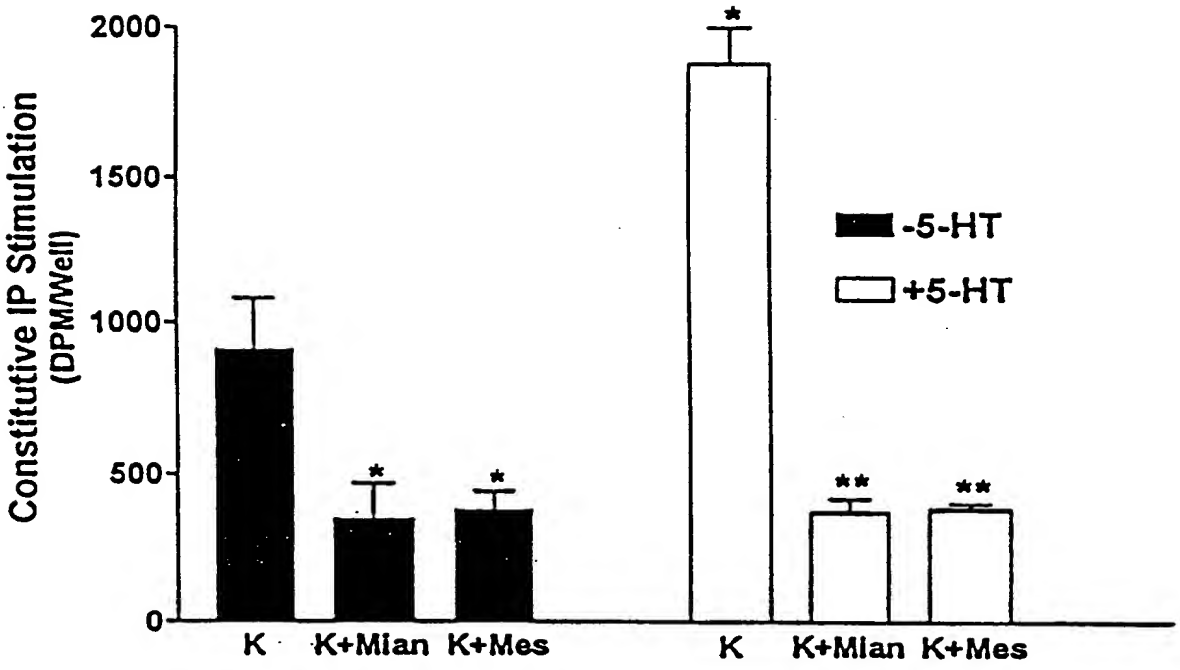

Constitutive IP Stimulation (DPM/Well)
2000
1500
1000
500
0
-5-HT
+5-HT
*
**
K
K+Mian
K+Mes

FIGURE 19

Human 5-HT_{2A}

1 gaattcgggt gagccagctc cgggagaaca gcatgtacac cagcctcagt gttacagagt

61 gtgggtacat caaggtgaat ggtgagcaga aactataacc tgttagtcct tctacacctc

121 atctgctaca agttctggct tagacatgga tattctttgt gaagaaaata cttctttgag

181 ctcaactacg aactccctaa tgcaattaaa tgatgacacc aggctctaca gtaatgactt

241 taactctgga gaagctaaca cttctgatgc atttaactgg acagtcgact ctgaaaatcg

301 aaccaacctt tcctgtgaag ggtgcctctc accgtcgtgt ctctccttac ttcatctcca

361 ggaaaaaaac tggtctgctt tactgacagc cgtagtgatt attctaacta ttgctggaaa

421 catactcgtc atcatggcag tgtccctaga gaaaaagctg cagaatgcca ccaactattt

481 cctgatgtca cttgccatag ctgatatgct gctgggtttc cttgtcatgc ccgtgtccat

541 gttaaccatc ctgtatgggt accggtggcc tctgccgagc aagctttgtg cagtctggat

601 ttacctggac gtgctcttct ccacggcctc catcatgcac ctctgcgcca tctcgctgga

661 ccgctacgtc gccatccaga atcccatcca ccacagccgc ttcaactcca gaactaaggc

721 atttctgaaa atcattgctg tttggaccat atcagtaggt atatccatgc caataccagt

781 ctttgggcta caggacgatt cgaaggtctt taaggagggg agttgcttac tcgccgatga

841 taactttgtc ctgatcggct cttttgtgtc atttttcatt cccttaacca tcatggtgat

901 cacctacttt ctaactatca agtcactcca gaaagaagct actttgtgtg taagtgatct

961 tggcacacgg gccaaattag cttctttcag cttcctccct cagagttctt tgtcttcaga

1021 aaagctcttc cagcggtcga tccataggga gccagggtcc tacacaggca ggaggactat

1081 gcagtccatc agcaatgagc aaaaggcatg caaggtgctg ggcatcgtct tcttcctgtt

1141 tgtggtgatg tggtgccctt tcttcatcac aaacatcatg gccgtcatct gcaaagagtc

1201 ctgcaatgag gatgtcattg gggccctgct caatgtgttt gtttggatcg gttatctctc

1261 ttcagcagtc aacccactag tctacacact gttcaacaag acctataggt cagccttttc

FIGURE 20A

1321 acggtatatt cagtgtcagt acaaggaaaa caaaaaacca ttgcagttaa ttttagtgaa

1381 cacaataccg gctttggcct acaagtctag ccaacttcaa atgggacaaa aaaagaattc

1441 aaagcaagat gccaagacaa cagataatga ctgctcaatg gttgctctag gaaagcagca

1501 ttctgaagag gcttctaaag acaatagcga cggagtgaat gaaaaggtga gctgtgtgtg

1561 ataggctagt tgccgtggca actgtggaag gcacactgag caagttttca cctatctgga

1621 aaaaaaaaat atgagattgg aaaaaattag acaagtctag tggaaccaac gatcatatct

1681 gtatgcctca ttttattctg tcaatgaaaa gcggggttca atgctacaaa atgtgtgctt

1741 ggaaaatgtt ctgacagcat ttcagctgtg agctttctga tacttattta taacattgta

1801 aatgatatgt ctttaaaatg attcactttt attgtataat tatgaagccc taagtaaatc

1861 taaattaact tctattttca agtggaaacc ttgctgctat gctgttcatt gatgacatgg

1921 gattgagttg gttacctatt gccgtaaata aaaatagcta taaatagtga aaatttttatt

1981 gaatataatg gcctcttaaa aattatcttt aaaacttact atggtatata ttttgaaagg

2041 agaaaaaaaa aaagccacta aggtcagtgt tataaaatct gtattgctaa gataattaaa

2101 tgaaatactt gacaacattt ttcatagata ccattttgaa atattcacaa ggttgctggc

2161 atttgctgca tttcaagtta attctcagaa gtgaaaaaga cttcaaatgt tattcaataa

2221 ctattgctgc tttctcttct acttcttgtg ctttactctg aatttccagt gtggtcttgt

2281 ttaatatttg ttcctctagg taaactagca aaaggatgat ttaacattac caaatgcctt

2341 tctagcaatt gcttctctaa aacagcacta tcgaggtatt tggtaacttg ctgtgaaatg

2401 actgcatcat gcatgcactc ttttgagcag taaatgtata ttgatgtaac tgtgtcagga

2461 ttgaggatga actcaggttt ccggctactg acagtggtag agtcctagga catctctgta

2521 aaaagcaggt gactttccta tgacactcat caggtaaact gatgctttca gatccatcgg

2581 tttatactat ttattaaaac cattctgctt ggttccacaa tcatctattg agtgtacatt

2641 tatgtgtgaa gcaaatttct agatatgaga aatataaaaa taattaaaac aaaatccttg

FIGURE 20A - CONTINUED

2701 ccttcaaacg aaatggctcg gccaggcacg gaggctcgtg catgtaatcc tagcactttg

2761 ggaggctgag atgggaggat cacttgaggc caagagtttg agaccaacct gggtaacaaa

2821 gtgagacctc cctgtctcta caaaaaaaat caaaaaatta tctgatcctt gtggcacaca

2881 actgtggtcc cagctacagg ggaggctgag acgcaaggat cacttgagcc cagaagctca

2941 aggctgcagt gagccaagtt cacaccactg ccatttcctc ctgggcaaca gagtgagacc

3001 ctatcacccc gaattc

FIGURE 20A - CONTINUED

Human 5-HT2A

MDILCEENTSLSSTTNSLMQLNDDTRLYSNDFNSGEANTSDAFN

WTVDSENRTNLSCEGCLSPSCLSLLHLQEKNWSALLTAVVIILTIAGNILVIMAVSLE

KKLQNATNYFLMSLAIADMLLGFLVMPVSMLTILYGYRWPLPSKLCAVWIYLDVLFST

ASIMHLCAISLDRYVAIQNPIHHSRFNSRTKAFLKIIAVWTISVGISMPIPVFGLQDD

SKVFKEGSCLLADDNFVLIGSFVSFFIPLTIMVITYFLTIKSLQKEATLCVSDLGTRA

KLASFSFLPQSSLSSEKLFQRSIHREPGSYTGRRTMQSISNEQKACKVLGIVFFLFVV

MWCPFFITNIMAVICKESCNEDVIGALLNVFVWIGYLSSAVNPLVYTLFNKTYRSAFS

RYIQCQYKENKKPLQLILVNTIPALAYKSSQLQMGQKKNSKQDAKTTDNDCSMVALGK

QHSEEASKDNSDGVNEKVSCV

FIGURE 20B

Human 5-HT_{2c}

1 gaattcggga gcgtcctcag atgcaccgat cttcccgata ctgcctttgg agcggctaga

61 ttgctagcct tggctgctcc attggcctgc cttgcccctt acctgccgat tgcatatgaa

121 ctcttcttct gtctgtacat cgttgtcgtc ggagtcgtcg cgatcgtcgt ggcgctcgtg

181 tgatggcctt cgtccgttta gagtagtgta gttagttagg ggccaacgaa gaagaaagaa

241 gacgcgatta gtgcagagat gctggaggtg gtcagttact aagctagagt aagatagcgg

301 agcgaaaaga gccaaaccta gccgggggggc gcacggtcac ccaaaggagg tcgactcgcc

361 ggcgcttcct atcgcgccga gctccctcca ttcctctccc tccgccgagg cgcgaggttg

421 cggcgcgcag cgcagcgcag ctcagcgcac cgactgccgc gggctccgct gggcgattgc

481 agccgagtcc gtttctcgtc tagctgccgc cgcggcgacc gctgcctggt cttcctcccg

541 gacgctagtg ggttatcagc taacacccgc gagcatctat aacataggcc aactgacgcc

601 atccttcaaa aacaactgtc tgggaaaaaa agaataaaaa gtagtgtgag agcagaaaac

661 gtgattgaaa cacgaccaat ctttcttcag tgccaaaggg tggaaaagaa aggatgatat

721 gatgaaccta gcctgttaat ttcgtcttct caatttttaaa ctttggttgc ttaagactga

781 agcaatcatg gtgaacctga ggaatgcggt gcattcattc cttgtgcacc taattggcct

841 attggtttgg caatgtgata tttctgtgag cccagtagca gctatagtaa ctgacatttt

901 caatacctcc gatggtggac gcttcaaatt cccagacggg gtacaaaact ggccagcact

961 ttcaatcgtc atcataataa tcatgacaat aggtggcaac atccttgtga tcatggcagt

1021 aagcatggaa aagaaactgc acaatgccac caattacttc ttaatgtccc tagccattgc

1081 tgatatgcta gtgggactac ttgtcatgcc cctgtctctc ctggcaatcc tttatgatta

1141 tgtctggcca ctacctagat atttgtgccc cgtctggatt tctttagatg ttttatttttc

1201 aacagcgtcc atcatgcacc tctgcgctat atcgctggat cggtatgtag caatacgtaa

1261 tcctattgag catagccgtt tcaattcgcg gactaaggcc atcatgaaga ttgctattgt

FIGURE 21A

1321 ttgggcaatt tctataggtg tatcagttcc tatccctgtg attggactga gggacgaaga

1381 aaaggtgttc gtgaacaaca cgacgtgcgt gctcaacgac ccaaatttcg ttcttattgg

1441 gtccttcgta gctttcttca taccgctgac gattatggtg attacgtatt gcctgaccat

1501 ctacgttctg cgccgacaag ctttgatgtt actgcacggc cacaccgagg aaccgcctgg

1561 actaagtctg gatttcctga agtgctgcaa gaggaatacg gccgaggaag agaactctgc

1621 aaaccctaac caagaccaga acgcacgccg aagaaaagaag aaggagagac gtcctagggg

1681 caccatgcag gctatcaaca atgaaagaaa agcttcgaaa gtccttggga ttgttttctt

1741 tgtgtttctg atcatgtggt gcccatttt cattaccaat attctgtctg ttctttgtga

1801 gaagtcctgt aaccaaaagc tcatggaaaa gcttctgaat gtgtttgttt ggattggcta

1861 tgtttgttca ggaatcaatc ctctggtgta tactctgttc aacaaaattt accgaagggc

1921 attctccaac tatttgcgtt gcaattataa ggtagagaaa aagcctcctg tcaggcagat

1981 tccaagagtt gccgccactg ctttgtctgg gagggagctt aatgttaaca tttatcggca

2041 taccaatgaa ccggtgatcg agaaagccag tgacaatgag cccggtatag agatgcaagt

2101 tgagaattta gagttaccag taaatccctc cagtgtggtt agcgaaagga ttagcagtgt

2161 gtgagaaaga acagcacagt cttttctacg gtacaagcta catatgtagg aaaatttct

2221 tctttaattt ttctgttggt cttaactaat gtaaatattg ctgtctgaaa aagtgttttt

2281 acatatagct ttgcaacctt gtactttaca atcatgccta cattagtgag atttagggtt

2341 ctatatttac tgtttataat aggtggagac taacttattt tgattgtttg atgaataaaa

2401 tgtttatttt tgctctccct cccttctttc cttccttttt tcctttcttc cttcctttct

2461 ctctttcttt tgtgcatatg gcaacgttca tgttcatctc aggtggcatt tgcaggtgac

2521 cagaatgagg cacatgacag tggttatatt tcaaccacac ctaaattaac aaattcagtg

2581 gacatttgtt ctgggttaac agtaaatata cactttacat tcttgctctg ctcatctaca

2641 catataaaca cagtaagata ggttctgctt tctgatacat ctgtcagtga gtcagaggca

FIGURE 21A - CONTINUED

2701 gaacctagtc ttgttgttca tataggggaa ttc

FIGURE 21A - CONTINUED

Human 5-HT_{2C}

MVNLRNAVHSFLVHLIGLLVWQCDISVSPVAAIVTDIFNTSDGG

RFKFPDGVQNWPALSIVIIIIMTIGGNILVIMAVSMEKKLHNATNYFLMSLAIADMLV

GLLVMPLSLLAILYDYVWPLPRYLCPVWISLDVLFSTASIMHLCAISLDRYVAIRNPI

EHSRFNSRTKAIMKIAIVWAISIGVSVPIPVIGLRDEEKVFVNNTTCVLNDPNFVLIG

SFVAFFIPLTIMVITYCLTIYVLRRQALMLLHGHTEEPPGLSLDFLKCCKRNTAEEEN

SANPNQDQNARRRKKKERRPRGTMQAINNERKASKVLGIVFFVFLIMWCPFFITNILS

VLCEKSCNQKLMEKLLNVFVWIGYVCSGINPLVYTLFNKIYRRAFSNYLRCNYKVEKK

PPVRQIPRVAATALSGRELNVNIYRHTNEPVIEKASDNEPGIEMQVENLELPVNPSSV

VSERISSV

FIGURE 21B

Rat 5-HT_{2A} Cysteine → Lysine Mutant

MEILCEDNISLSSIPNSLMQLGDGPRLYHNDFNSRDANTSEASN

WTIDAENRTNLSCEGYLPPTCLSILHLQEKNWSALLTTVVIILTIAGNILVIMAVSLE

KKLQNATNYFLMSLAIADMLLGFLVMPVSMLTILYGYRWPLPSKLCAIWIYLDVLFST

ASIMHLCAISLDRYVAIQNPIHHSRFNSRTKAFLKIIAVWTISVGISMPIPVFGLQDD

SKVFKEGSCLLADDNFVLIGSFVAFFIPLTIMVITYFLTIKSLQKEATLCVSDLSTRA

KLASFSFLPQSSLSSEKLFQRSIHREPGSYAGRRTMQSISNEQKA[K]KVLGIVFFLFVV

MWCPFFITNIMAVICKESCNENVIGALLNVFVWIGYLSSAVNPLVYTLFNKTYRSAFS

RYIQCQYKENRKPLQLILVNTIPALAYKSSQLQVGQKKNSQEDAEQTVDDCSMVTLGK

QQSEENCTDNIETVNEKVSCV

FIGURE 22

Rat $5HT_{2A}$ Cysteine → Lysine Mutant

1 cccaggctat gaacccctag tctctccaca cttcatctgc tacaacttcc ggcttagaca

61 tggaaattct ttgtgaagac aatatctctc tgagctcaat tccaaactcc ttaatgcaat

121 taggtgatgg cccgaggctc taccataatg acttcaactc cagagatgct aacacttcgg

181 aagcatcgaa ctggacaatt gatgctgaaa acagaaccaa cctctcctgt gaagggtacc

241 tcccaccgac atgcctctcc attcttcatc tccaggaaaa aaactggtct gctttattga

301 caactgtcgt gattattctc accattgctg gaaatatact ggtcatcatg gcagtgtccc

361 tagaaaaaaa gctgcagaat gccaccaact atttcctgat gtcacttgcc atagctgata

421 tgctgctggg tttccttgtc atgcctgtgt ccatgttaac catcctgtat gggtaccggt

481 ggcctttgcc tagcaagctc tgtgcgatct ggatttacct ggatgtgctc ttttctacgg

541 catccatcat gcacctctgc gccatctccc tggaccgcta tgtcgccatc cagaacccca

601 ttcaccacag ccgcttcaac tccagaacca aagccttcct gaaaatcatt gccgtgtgga

661 ccatatctgt aggtatatcc atgccaatcc cagtctttgg actacaggat gattcgaagg

721 tctttaagga ggggagctgc ctgcttgccg atgacaactt tgttctcata ggctcttttg

781 tggcattttt catccccctа accatcatgg tgatcaccta cttcctgact atcaagtcac

841 ttcagaaaga agccacctta tgtgtgagtg acctcagcac tcgagccaaa ctagcctcct

901 tcagcttcct ccctcagagt tctctgtcat cagaaaagct cttccaacgg tccatccaca

Start C322K primer

961 gagagccagg ctcctacgca ggccgaagga cgatgcagtc catcagcaat gagcaaaagg

End C322K primer

1021 cgaaggaaggt gctgggcatc gtgttcttcc tgtttgttgt aatgtggtgc ccattcttca

1081 tcaccaatat catggccgtc atctgcaaag aatcctgcaa tgaaaatgtc atcggagccc

1141 tgctcaatgt gtttgtctgg attggttatc tctcctcagc tgtcaatcca ctggtatata

1201 cgttgttcaa taaaacttat aggtccgcct tctcaaggta cattcagtgt cagtacaagg

1261 aaaacagaaa gccactgcag ttaattttag tgaacactat accagcattg gcctacaagt

FIGURE 23

1321 ctagtcagct ccaggtggga cagaaaaaga actcacagga agatgctgag cagacagttg

1381 atgactgctc catggttaca ctggggaaac aacagtcgga agagaattgt acagacaata

1441 ttgaaaccgt gaatgaaaag gttagctgtg tgtgatgaac tggatgctat ggcaattgcc

1501 cagggcatgt gaacaaggtt atacccatgt gtgtggggcg gggataagga ggctgcaaca

1561 aattag

FIGURE 23 - CONTINUED

Rat $5HT_{2A}$ Cysteine → Lysine Mutant with Restriction Site

1 cccaggctat gaacccctag tctctccaca cttcatctgc tacaacttcc ggcttagaca

61 tggaaattct ttgtgaagac aatatctctc tgagctcaat tccaaactcc ttaatgcaat

121 taggtgatgg cccgaggctc taccataatg acttcaactc cagagatgct aacacttcgg

181 aagcatcgaa ctggacaatt gatgctgaaa acagaaccaa cctctcctgt gaagggtacc

241 tcccaccgac atgcctctcc attcttcatc tccaggaaaa aaactggtct gctttattga

301 caactgtcgt gattattctc accattgctg gaaatatact ggtcatcatg gcagtgtccc

361 tagaaaaaaa gctgcagaat gccaccaact atttcctgat gtcacttgcc atagctgata

421 tgctgctggg tttccttgtc atgcctgtgt ccatgttaac catcctgtat gggtaccggt

481 ggcctttgcc tagcaagctc tgtgcgatct ggatttacct ggatgtgctc ttttctacgg

541 catccatcat gcacctctgc gccatctccc tggaccgcta tgtcgccatc cagaacccca

601 ttcaccacag ccgcttcaac tccagaacca aagccttcct gaaaatcatt gccgtgtgga

661 ccatatctgt aggtatatcc atgccaatcc cagtctttgg actacaggat gattcgaagg

721 tctttaagga ggggagctgc ctgcttgccg atgacaactt tgttctcata ggctcttttg

781 tggcattttt catcccccta accatcatgg tgatcaccta cttcctgact atcaagtcac

841 ttcagaaaga agccacctta tgtgtgagtg acctcagcac tcgagccaaa ctagcctcct

901 tcagcttcct ccctcagagt tctctgtcat cagaaaagct cttccaacgg tccatccaca

Start C322K primer

961 gagagccagg ctcctacgca ggccgaagga cgatgcagtc catcagcaat gagcaaaagg

End C322K primer

1021 cgaaagaaagt actgggcatc gtgttcttcc tgtttgttgt aatgtggtgc ccattcttca

Mutations to create Sca1 site

1081 tcaccaatat catggccgtc atctgcaaag aatcctgcaa tgaaaatgtc atcggagccc

1141 tgctcaatgt gtttgtctgg attggttatc tctcctcagc tgtcaatcca ctggtatata

1201 cgttgttcaa taaaacttat aggtccgcct tctcaaggta cattcagtgt cagtacaagg

1261 aaaacagaaa gccactgcag ttaattttag tgaacactat accagcattg gcctacaagt

FIGURE 24

1321 ctagtcagct ccaggtggga cagaaaaaga actcacagga agatgctgag cagacagttg

1381 atgactgctc catggttaca ctggggaaac aacagtcgga agagaattgt acagacaata

1441 ttgaaaccgt gaatgaaaag gttagctgtg tgtgatgaac tggatgctat ggcaattgcc

1501 cagggcatgt gaacaaggtt atacccatgt gtgtggggcg gggataagga ggctgcaaca

1561 aattag

FIGURE 24 - CONTINUED

Rat 5-HT_{2A} Cysteine → Arginine Mutant

MEILCEDNISLSSIPNSLMQLGDGPRLYHNDFNSRDANTSEASN

WTIDAENRTNLSCEGYLPPTCLSILHLQEKNWSALLTTVVIILTIAGNILVIMAVSLE

KKLQNATNYFLMSLAIADMLLGFLVMPVSMLTILYGYRWPLPSKLCAIWIYLDVLFST

ASIMHLCAISLDRYVAIQNPIHHSRFNSRTKAFLKIIAVWTISVGISMPIPVFGLQDD

SKVFKEGSCLLADDNFVLIGSFVAFFIPLTIMVITYFLTIKSLQKEATLCVSDLSTRA

KLASFSFLPQSSLSSEKLFQRSIHREPGSYAGRRTMQSISNEQKA[R]KVLGIVFFLFVV

MWCPFFITNIMAVICKESCNENVIGALLNVFVWIGYLSSAVNPLVYTLFNKTYRSAFS

RYIQCQYKENRKPLQLILVNTIPALAYKSSQLQVGQKKNSQEDAEQTVDDCSMVTLGK

QQSEENCTDNIETVNEKVSCV

FIGURE 25

Rat $5HT_{2A}$ Cysteine → Arginine Mutant

1 cccaggctat gaacccctag tctctccaca cttcatctgc tacaacttcc ggcttagaca

61 tggaaattct ttgtgaagac aatatctctc tgagctcaat tccaaactcc ttaatgcaat

121 taggtgatgg cccgaggctc taccataatg acttcaactc cagagatgct aacacttcgg

181 aagcatcgaa ctggacaatt gatgctgaaa acagaaccaa cctctcctgt gaagggtacc

241 tcccaccgac atgcctctcc attcttcatc tccaggaaaa aaactggtct gctttattga

301 caactgtcgt gattattctc accattgctg gaaatatact ggtcatcatg gcagtgtccc

361 tagaaaaaaa gctgcagaat gccaccaact atttcctgat gtcacttgcc atagctgata

421 tgctgctggg tttccttgtc atgcctgtgt ccatgttaac catcctgtat gggtaccggt

481 ggcctttgcc tagcaagctc tgtgcgatct ggatttacct ggatgtgctc ttttctacgg

541 catccatcat gcacctctgc gccatctccc tggaccgcta tgtcgccatc cagaacccca

601 ttcaccacag ccgcttcaac tccagaacca aagccttcct gaaaatcatt gccgtgtgga

661 ccatatctgt aggtatatcc atgccaatcc cagtctttgg actacaggat gattcgaagg

721 tctttaagga ggggagctgc ctgcttgccg atgacaactt tgttctcata ggctcttttg

781 tggcattttt catccccctа accatcatgg tgatcaccta cttcctgact atcaagtcac

841 ttcagaaaga agccacctig tgtgtgagtg acctcagcac tcgagccaaa ctagcctcct

901 tcagcttcct ccctcagagt tctctgtcat cagaaaagct cttccaacgg tccatccaca

Start C322R primer

961 gagagccagg ctcctacgca ggccgaagga cgatgcagtc catcagcaat gagcaaaagg

End C322R primer

1021 cgagggaaggt gctgggcatc gtgttcttcc tgtttgttgt aatgtggtgc ccattcttca

1081 tcaccaatat catggccgtc atctgcaaag aatcctgcaa tgaaaatgtc atcggagccc

1141 tgctcaatgt gtttgtctgg attggttatc tctcctcagc tgtcaatcca ctggtatata

1201 cgttgttcaa taaaacttat aggtccgcct tctcaaggta cattcagtgt cagtacaagg

1261 aaaacagaaa gccactgcag ttaattttag tgaacactat accagcattg gcctacaagt

FIGURE 26

1321 ctagtcagct ccaggtggga cagaaaaaga actcacagga agatgctgag cagacagttg

1381 atgactgctc catggttaca ctggggaaac aacagtcgga agagaattgt acagacaata

1441 ttgaaaccgt gaatgaaaag gttagctgtg tgtgatgaac tggatgctat ggcaattgcc

1501 cagggcatgt gaacaaggtt atacccatgt gtgtggggcg gggataagga ggctgcaaca

1561 aattag

FIGURE 26 - CONTINUED

Rat $5HT_{2A}$ Cysteine → Arginine Mutant with Restriction Site

1 cccaggctat gaacccctag tctctccaca cttcatctgc tacaacttcc ggcttagaca

61 tggaaattct ttgtgaagac aatatctctc tgagctcaat tccaaactcc ttaatgcaat

121 taggtgatgg cccgaggctc taccataatg acttcaactc cagagatgct aacacttcgg

181 aagcatcgaa ctggacaatt gatgctgaaa acagaaccaa cctctcctgt gaagggtacc

241 tcccaccgac atgcctctcc attcttcatc tccaggaaaa aaactggtct gctttattga

301 caactgtcgt gattattctc accattgctg gaaatatact ggtcatcatg gcagtgtccc

361 tagaaaaaaa gctgcagaat gccaccaact atttcctgat gtcacttgcc atagctgata

421 tgctgctggg tttccttgtc atgcctgtgt ccatgttaac catcctgtat gggtaccggt

481 ggcctttgcc tagcaagctc tgtgcgatct ggatttacct ggatgtgctc ttttctacgg

541 catccatcat gcacctctgc gccatctccc tggaccgcta tgtcgccatc cagaacccca

601 ttcaccacag ccgcttcaac tccagaacca aagccttcct gaaaatcatt gccgtgtgga

661 ccatatctgt aggtatatcc atgccaatcc cagtctttgg actacaggat gattcgaagg

721 tctttaagga ggggagctgc ctgcttgccg atgacaactt tgttctcata ggctcttttg

781 tggcattttt catcccccta accatcatgg tgatcaccta cttcctgact atcaagtcac

841 ttcagaaaga agccaccttg tgtgtgagtg acctcagcac tcgagccaaa ctagcctcct

901 tcagcttcct ccctcagagt tctctgtcat cagaaaagct cttccaacgg tccatccaca

Start C322R primer

961 gagagccagg ctcctacgca ggccgaagga cgatgcagtc catcagcaat gagcaaaagg

End C322R primer

1021 cgaggaaggt gctgggcatc gtgttcttcc tgtttgttgt aatgtggtgc ccattcttca

1081 tcaccaatat catggccgtc atctgcaaag aatcctgcaa tgaaaatgtc atcggagccc

1141 tgctcaatgt gtttgtctgg attggttatc tctcctcagc tgtcaatcca ctggtatata

1201 cgttgttcaa taaaacttat aggtccgcct tctcaaggta cattcagtgt cagtacaagg

1261 aaaacagaaa gccactgcag ttaattttag tgaacactat accagcattg gcctacaagt

FIGURE 27

1321 ctagtcagct ccaggtggga cagaaaaaga actcacagga agatgctgag cagacagttg

1381 atgactgctc catggttaca ctggggaaac aacagtcgga agaggaattgt acagacaata

1441 ttgaaaccgt gaatgaaaag gttagctgtg tgtgatgaac tggatgctat ggcaattgcc

1501 cagggcatgt gaacaaggtt atacccatgt gtgtggggcg gggataagga ggctgcaaca

1561 aattag

FIGURE 27 - CONTINUED

Rat 5-HT_{2A} Cysteine → Glutamic Acid Mutant

MEILCEDNISLSSIPNSLMQLGDGPRLYHNDFNSRDANTSEASN

WTIDAENRTNLSCEGYLPPTCLSILHLQEKNWSALLTTVVIILTIAGNILVIMAVSLE

KKLQNATNYFLMSLAIADMLLGFLVMPVSMLTILYGYRWPLPSKLCAIWIYLDVLFST

ASIMHLCAISLDRYVAIQNPIHHSRFNSRTKAFLKIIAVWTISVGISMPIPVFGLQDD

SKVFKEGSCLLADDNFVLIGSFVAFFIPLTIMVITYFLTIKSLQKEATLCVSDLSTRA

KLASFSFLPQSSLSSEKLFQRSIHREPGSYAGRRTMQSISNEQKA[E]KVLGIVFFLFVV

MWCPFFITNIMAVICKESCNENVIGALLNVFVWIGYLSSAVNPLVYTLFNKTYRSAFS

RYIQCQYKENRKPLQLILVNTIPALAYKSSQLQVGQKKNSQEDAEQTVDDCSMVTLGK

QQSEENCTDNIETVNEKVSCV

FIGURE 28

Rat $5HT_{2A}$ Cysteine → Glutamic Acid Mutant

1 cccaggctat gaacccctag tctctccaca cttcatctgc tacaacttcc ggcttagaca

61 tggaaattct ttgtgaagac aatatctctc tgagctcaat tccaaactcc ttaatgcaat

121 taggtgatgg cccgaggctc taccataatg acttcaactc cagagatgct aacacttcgg

181 aagcatcgaa ctggacaatt gatgctgaaa acagaaccaa cctctcctgt gaagggtacc

241 tcccaccgac atgcctctcc attcttcatc tccaggaaaa aaactggtct gctttattga

301 caactgtcgt gattattctc accattgctg gaaatatact ggtcatcatg gcagtgtccc

361 tagaaaaaaa gctgcagaat gccaccaact atttcctgat gtcacttgcc atagctgata

421 tgctgctggg tttccttgtc atgcctgtgt ccatgttaac catcctgtat gggtaccggt

481 ggcctttgcc tagcaagctc tgtgcgatct ggatttacct ggatgtgctc ttttctacgg

541 catccatcat gcacctctgc gccatctccc tggaccgcta tgtcgccatc cagaacccca

601 ttcaccacag ccgcttcaac tccagaacca aagccttcct gaaaatcatt gccgtgtgga

661 ccatatctgt aggtatatcc atgccaatcc cagtctttgg actacaggat gattcgaagg

721 tctttaagga ggggagctgc ctgcttgccg atgacaactt tgttctcata ggctcttttg

781 tggcattttt catccccctа accatcatgg tgatcaccta cttcctgact atcaagtcac

841 ttcagaaaga agccaccttg tgtgtgagtg acctcagcac tcgagccaaa ctagcctcct

901 tcagcttcct ccctcagagt tctctgtcat cagaaaagct cttccaacgg tccatccaca

Start C322E primer

961 gagagccagg ctcctacgca ggccgaagga cgatgcagtc catcagcaat gagcaaaagg

End C322E primer

1021 cggaggaaggt gctgggcatc gtgttcttcc tgtttgttgt aatgtggtgc ccattcttca

1081 tcaccaatat catggccgtc atctgcaaag aatcctgcaa tgaaaatgtc atcggagccc

1141 tgctcaatgt gtttgtctgg attggttatc tctcctcagc tgtcaatcca ctggtatata

1201 cgttgttcaa taaaacttat aggtccgcct tctcaaggta cattcagtgt cagtacaagg

1261 aaaacagaaa gccactgcag ttaattttag tgaacactat accagcattg gcctacaagt

FIGURE 29

1321 ctagtcagct ccaggtggga cagaaaaaga actcacagga agatgctgag cagacagttg

1381 atgactgctc catggttaca ctggggaaac aacagtcgga agagaattgt acagacaata

1441 ttgaaaccgt gaatgaaaag gttagctgtg tgtgatgaac tggatgctat ggcaattgcc

1501 cagggcatgt gaacaaggtt atacccatgt gtgtggggcg gggataagga ggctgcaaca

1561 aattag

FIGURE 29 - CONTINUED

Rat $5HT_{2A}$ Cysteine → Glutamic Acid Mutant with Restriction Site

1 cccaggctat gaacccctag tctctccaca cttcatctgc tacaacttcc ggcttagaca

61 tggaaattct ttgtgaagac aatatctctc tgagctcaat tccaaactcc ttaatgcaat

121 taggtgatgg cccgaggctc taccataatg acttcaactc cagagatgct aacacttcgg

181 aagcatcgaa ctggacaatt gatgctgaaa acagaaccaa cctctcctgt gaagggtacc

241 tcccaccgac atgcctctcc attcttcatc tccaggaaaa aaactggtct gctttattga

301 caactgtcgt gattattctc accattgctg gaaatatact ggtcatcatg gcagtgtccc

361 tagaaaaaaa gctgcagaat gccaccaact atttcctgat gtcacttgcc atagctgata

421 tgctgctggg tttccttgtc atgcctgtgt ccatgttaac catcctgtat gggtaccggt

481 ggcctttgcc tagcaagctc tgtgcgatct ggatttacct ggatgtgctc ttttctacgg

541 catccatcat gcacctctgc gccatctccc tggaccgcta tgtcgccatc cagaacccca

601 ttcaccacag ccgcttcaac tccagaacca aagccttcct gaaaatcatt gccgtgtgga

661 ccatatctgt aggtatatcc atgccaatcc cagtctttgg actacaggat gattcgaagg

721 tctttaagga ggggagctgc ctgcttgccg atgacaactt tgttctcata ggctcttttg

781 tggcattttt catcccccta accatcatgg tgatcaccta cttcctgact atcaagtcac

841 ttcagaaaga agccaccttg tgtgtgagtg acctcagcac tcgagccaaa ctagcctcct

901 tcagcttcct ccctcagagt tctctgtcat cagaaaagct cttccaacgg tccatccaca

Start C322E primer

961 gagagccagg ctcctacgca ggccgaagga cgatgcagtc catcagcaat gagcaaaagg

End C322E primer

1021 cgggaaggt actgggcatc gtgttcttcc tgtttgttgt aatgtggtgc ccattcttca

Mutation to create Rsa1 site

1081 tcaccaatat catggccgtc atctgcaaag aatcctgcaa tgaaaatgtc atcggagccc

1141 tgctcaatgt gtttgtctgg attggttatc tctcctcagc tgtcaatcca ctggtatata

1201 cgttgttcaa taaaacttat aggtccgcct tctcaaggta cattcagtgt cagtacaagg

1261 aaaacagaaa gccactgcag ttaattttag tgaacactat accagcattg gcctacaagt

FIGURE 30

1321 ctagtcagct ccaggtggga cagaaaaaga actcacagga agatgctgag cagacagttg

1381 atgactgctc catggttaca ctggggaaac aacagtcgga agagaattgt acagacaata

1441 ttgaaaccgt gaatgaaaag gttagctgtg tgtgatgaac tggatgctat ggcaattgcc

1501 cagggcatgt gaacaaggtt atacccatgt gtgtggggcg gggataagga ggctgcaaca

1561 aattag

FIGURE 30 - CONTINUED

Rat 5-HT_{2C} Serine → Lysine Mutant

MVNLGNAVRSLLMHLIGLLVWQFDISISPVAAIVTDTFNSSDGG

RLFQFPDGVQNWPALSIVVIIIMTIGGNILVIMAVSMEKKLHNATNYFLMSLAIADML

VGLLVMPLSLLAILYDYVWPLPRYLCPVWISLDVLFSTASIMHLCAISLDRYVAIRNP

IEHSRFNSRTKAIMKIAIVWAISIGVSVPIPVIGLRDESKVFVNNTTCVLNDPNFVLI

GSFVAFFIPLTIMVITYFLTIYVLRRQTLMLLRGHTEEELANMSLNFLNCCCKKNGGE

EENAPNPNPDQKPRRKKKEKRPRGTMQAINNEKKAKKVLGIVFFVFLIMWCPFFITNI

LSVLCGKACNQKLMEKLLNVFVWIGYVCSGINPLVYTLFNKIYRRAFSKYLRCDYKPD

KKPPVRQIPRVAATALSGRELNVNIYRHTNERVARKANDPEPGIEMQVENLELPVNPS

NVVSERISSV

FIGURE 31

Rat $5HT_{2C}$ Serine → Lysine Mutant

ORIGIN 23 bp upstream of HindIII site.

1 ggcgctctgg tgctcactga ggaagcttcc ttaggtgtac cgatcttaat gattgagccc

61 ttggagcagc aagattgtta atcttggttg ctcctttggc ctgtctatcc cttaccttcc

121 tattacatat gaacttttct tcgttctgca catcgattgt cgtcggcgtc gtggagatcg

181 tcgtggtgct ccggtggtgg tcttcgtccg cttagaatag tgtagttagt taggggcctt

241 caaagaagaa agaagaagcg attggcgcgg agagatgctg gaggtgtcag tttctatgct

301 agagtagggt agtgaaacaa tccccagcca aacctttccg gggggcgcag gttgcccaca

361 ggaggtcgac ttgccggcgc tgtccttcgc gccgagctcc ctccatcctt ctttccgtct

421 gctgagacgc aaggttgcgg cgcgcacgct gagcagcgca ctgactgccg cgggctccgc

481 tgggcgattg cagccgagtc cgtttctcgt ctagctgccg ccgcggcgac ctgcctggtc

541 ttcctcccgg acgctagcgg gttgtcaact attacctgca agcataggcc aacgaacacc

601 ttctttccaa attaattgga atgaaacaat tctgttaact tcctaattct cagtttgaaa

661 ctctggttgc ttaagcctga agcaatcatg gtgaaccttg gcaacgcggt gcgctcgctc

721 ctgatgcacc taatcggcct attggtttgg caattcgata tttccataag tccagtagca

781 gctatagtaa ctgacacttt taattcctcc gatggtggac gcttgtttca attcccggac

841 ggggtacaaa actggccagc actttcaatc gtcgtgatta taatcatgac aatagggggc

901 aacattcttg ttatcatggc agtaagcatg gagaagaaac tgcacaatgc aaccaattac

961 ttcttaatgt ccctagccat tgctgatatg ctggtgggac tacttgtcat gcccctgtcc

1021 ctgcttgcta ttctttatga ttatgtctgg cctttaccta gatatttgtg ccccgtctgg

1081 atttcactag atgtgctatt ttcaactgcg tccatcatgc acctctgcgc catatcgctg

1141 gaccggtatg tagcaatacg taatcctatt gagcatagcc ggttcaattc gcggactaag

1201 gccatcatga agattgccat cgtttgggca atatcaatag gagtttcagt tcctatccct

FIGURE 32

1261 gtgattggac tgagggacga aagcaaagtg ttcgtgaata acaccacgtg cgtgctcaat

1321 gaccccaact tcgttctcat cgggtccttc gtggcattct tcatcccgtt gacgattatg

1381 gtgatcacct acttcttaac gatctacgtc ctgcgccgtc aaactctgat gttacttcga

1441 ggtcacaccg aggaggaact ggctaatatg agcctgaact ttctgaactg ctgctgcaag

1501 aagaatggtg gtgaggaaga gaacgctccg aaccctaatc cagatcagaa accacgtcga

Start S312K primer

1561 aagaagaaag aaaagcgtcc cagaggcacc atgcaagcta tcaacaacga aaagaaagct

End S312K primer

1621 aaggaaagtcc ttggcattgt attctttgtg tttctgatca tgtggtgccc gtttttcatc

1681 accaatatcc tgtcggttct ttgtgggaag gcctgtaacc aaaagctaat ggagaagctt

1741 ctcaatgtgt ttgtgtggat tggctatgtg tgttcaggca tcaatcctct ggtgtacact

1801 ctctttaata aaatttaccg aagggctttc tctaaatatt tgcgctgcga ttataagcca

1861 gacaaaaagc ctcctgttcg acagattcct agggttgctg ccactgcttt gtctgggagg

1921 gagctcaatg ttaacattta tcggcatacc aatgaacgtg tggctaggaa agctaatgac

1981 cctgagcctg gcatagagat gcaggtggag aacttagagc tgccagtcaa cccctctaat

2041 gtggtcagcg agaggattag tagtgtgtaa gcgaagagca gcgcagactt cctacaggaa

2101 agttcctgta ggaaagtcct ccccaccccc cgtgattttc ctgtgaatca taactaatgt

2161 aaatattgct gtgtgacaag acagtgtttt tataaatagc tttgcaaccc tgtactttac

2221 atcatgcgtt aatagtgaga ttcggg

FIGURE 32 - CONTINUED

Rat $5HT_{2C}$ Serine → Lysine Mutant with Restriction Site

ORIGIN 23 bp upstream of HindIII site.

1 ggcgctctgg tgctcactga ggaagcttcc ttaggtgtac cgatcttaat gattgagccc

61 ttggagcagc aagattgtta atcttggttg ctcctttggc ctgtctatcc cttaccttcc

121 tattacatat gaacttttct tcgttctgca catcgattgt cgtcggcgtc gtggagatcg

181 tcgtggtgct ccggtggtgg tcttcgtccg cttagaatag tgtagttagt taggggcctt

241 caaagaagaa agaagaagcg attggcgcgg agagatgctg gaggtgtcag tttctatgct

301 agagtagggt agtgaaacaa tccccagcca aacctttccg gggggcgcag gttgcccaca

361 ggaggtcgac ttgccggcgc tgtccttcgc gccgagctcc ctccatcctt ctttccgtct

421 gctgagacgc aaggttgcgg cgcgcacgct gagcagcgca ctgactgccg cgggctccgc

481 tgggcgattg cagccgagtc cgtttctcgt ctagctgccg ccgcggcgac ctgcctggtc

541 ttcctcccgg acgctagcgg gttgtcaact attacctgca agcataggcc aacgaacacc

601 ttctttccaa attaattgga atgaaacaat tctgttaact tcctaattct cagtttgaaa

661 ctctggttgc ttaagcctga agcaatcatg gtgaaccttg gcaacgcggt gcgctcgctc

721 ctgatgcacc taatcggcct attggtttgg caattcgata tttccataag tccagtagca

781 gctatagtaa ctgacacttt taattcctcc gatggtggac gcttgtttca attcccggac

841 ggggtacaaa actggccagc actttcaatc gtcgtgatta taatcatgac aatagggggc

901 aacattcttg ttatcatggc agtaagcatg gagaagaaac tgcacaatgc aaccaattac

961 ttcttaatgt ccctagccat tgctgatatg ctggtgggac tacttgtcat gcccctgtcc

1021 ctgcttgcta ttctttatga ttatgtctgg cctttaccta gatatttgtg ccccgtctgg

1081 atttcactag atgtgctatt ttcaactgcg tccatcatgc acctctgcgc catatcgctg

1141 gaccggtatg tagcaatacg taatcctatt gagcatagcc ggttcaattc gcggactaag

1201 gccatcatga agattgccat cgtttgggca atatcaatag gagtttcagt tcctatccct

FIGURE 33

1261 gtgattggac tgagggacga aagcaaagtg ttcgtgaata acaccacgtg cgtgctcaat

1321 gaccccaact tcgttctcat cgggtccttc gtggcattct tcatccccgtt gacgattatg

1381 gtgatcacct acttcttaac gatctacgtc ctgcgccgtc aaactctgat gttacttcga

1441 ggtcacaccg aggaggaact ggctaatatg agcctgaact ttctgaactg ctgctgcaag

1501 aagaatggtg gtgaggaaga gaacgctccg aaccctaatc cagatcagaa accacgtcga

Start S312K primer

1561 aagaagaaag aaaagcgtcc cagaggcacc atgcaagcta tcaacaacga aaagaaagct

End S312K primer

1621 aaggaaagtac ttggcattgt attctttgtg tttctgatca tgtggtgccc gtttttcatc

Mutation to create Sca1 site

1681 accaatatcc tgtcggttct ttgtgggaag gcctgtaacc aaaagctaat ggagaagctt

1741 ctcaatgtgt ttgtgtggat tggctatgtg tgttcaggca tcaatcctct ggtgtacact

1801 ctctttaata aaatttaccg aagggctttc tctaaatatt tgcgctgcga ttataagcca

1861 gacaaaaagc ctcctgttcg acagattcct agggttgctg ccactgcttt gtctgggagg

1921 gagctcaatg ttaacattta tcggcatacc aatgaacgtg tggctaggaa agctaatgac

1981 cctgagcctg gcatagagat gcaggtggag aacttagagc tgccagtcaa cccctctaat

2041 gtggtcagcg agaggattag tagtgtgtaa gcgaagagca gcgcagactt cctacaggaa

2101 agttcctgta ggaaagtcct ccccaccccc cgtgattttc ctgtgaatca taactaatgt

2161 aaatattgct gtgtgacaag acagtgtttt tataaatagc tttgcaaccc tgtactttac

2221 atcatgcgtt aatagtgaga ttcggg

FIGURE 33 - CONTINUED

Rat 5-HT_{2C} Serine → Phenylalanine Mutant

MVNLGNAVRSLLMHLIGLLVWQFDISISPVAAIVTDTFNSSDGG

RLFQFPDGVQNWPALSIVVIIIMTIGGNILVIMAVSMEKKLHNATNYFLMSLAIADML

VGLLVMPLSLLAILYDYVWPLPRYLCPVWISLDVLFSTASIMHLCAISLDRYVAIRNP

IEHSRFNSRTKAIMKIAIVWAISIGVSVPIPVIGLRDESKVFVNNTTCVLNDPNFVLI

GSFVAFFIPLTIMVITYFLTIYVLRRQTLMLLRGHTEEELANMSLNFLNCCCKKNGGE

EENAPNPNPDQKPRRKKKEKRPRGTMQAINNEKKAFKVLGIVFFVFLIMWCPFFITNI

LSVLCGKACNQKLMEKLLNVFVWIGYVCSGINPLVYTLFNKIYRRAFSKYLRCDYKPD

KKPPVRQIPRVAATALSGRELNVNIYRHTNERVARKANDPEPGIEMQVENLELPVNPS

NVVSERISSV

FIGURE 34